

OPSONIZATION OF STREPTOCOCCUS PNEUMONIAE

With particular reference to vaccine induced antibodies

and deficiency of properdin

Jean Henrik Braconier
—

Lund 1983
— —

J H Braconier

**Department of Infectious Diseases and
Research Department 2, University of Lund , Sweden**

1983



Tryckt på Publica-tryckeriet, Lund 1983

CONTENTS

INTRODUCTION	5
Historical aspects	5
<u>S. pneumoniae</u>	7
Current clinical importance of <u>S. pneumoniae</u>	9
Host defence	11
THE PRESENT INVESTIGATION	29
MATERIALS AND METHODS	30
RESULTS	33
Opsonophagocytic killing of <u>S. pneumoniae</u> (I)	33
Properdin deficiency. Description of a family, influence by properdin deficiency on alternative complement path- way function and on pneumococcal opsonization (II, III, IV)	39
Opsonic antibodies after pneumococcal vaccination of immuno- compromised patients (V)	45
Cross-reacting opsonic antibodies after pneumococcal vaccination (VI)	49
SUMMARY	52

ACKNOWLEDGEMENTS

54

REFERENCES

56

APPENDIX: Papers I-VI

The present thesis is based on the following papers:

- I. Braconier, J.H. and Odeberg, H.: Granulocyte phagocytosis and killing of virulent and avirulent serotypes of Streptococcus pneumoniae. J Lab Clin Med 100:279-287, 1982.
- II. Sjöholm, A.G., Braconier, J.H. and Söderström, C.: Properdin deficiency in a family with fulminant meningococcal infections. Clin Exp Immunol 50:291-297, 1982.
- III. Braconier, J.H., Sjöholm, A.G. and Söderström, C.: Fulminant meningococcal infections in a family with inherited deficiency of properdin.
Accepted for publication in Scand J Infect Dis.
- IV. Braconier, J.H., Odeberg, H. and Sjöholm, A.G.: Granulocyte phagocytosis of Streptococcus pneumoniae in properdin deficient serum. Submitted to Infect Immun.
- V. Braconier, J.H., Karup Pedersen, F., Odeberg, H. and Rosén, C.: Opsonic and antibody responses to pneumococcal polysaccharide types 6A, 19F and 23F after vaccination of immunocompromized patients. Accepted for publication in Scand J Infect Dis.

- VI. Braconier, J.H., Myhre, E.B. and Odeberg, H.: Pneumococcal vaccination. Opsonic and antibody response to clinically important serotypes, not included in the vaccine. Submitted to J Infect Dis.

Individual papers will be referred to by their Roman numerals.

INTRODUCTION

Historical aspects (c f White 1979). The first to describe the pneumococcus was probably Klebs in 1875, who found the organism in fluid from lungs of patients dying in pneumonia. Pasteur and Sternberg showed in 1881 the infectiousity of the bacteria and Friedländer could cultivate the organisms. Through Frankel's experiments in 1886, the postulates of Koch's law were accomplished. He also named the organisms pneumococcus. The first successful animal immunizations were performed in 1891 by Klemperer and the protective effect of immune serum was discovered some years later by Denys.

By the use of serological methods, Neufeld and Haendel defined distinct serotypes of the pneumococci. The detection of various serotypes was an important basis for efficient production of anti-pneumococcal serum and its use in the treatment of severe pneumococcal infections. The development of vaccines to pneumococci was also founded on knowledge of different serotypes.

Before the era of antibiotics, pneumococcal pneumonia had a mortality of about 20-30 % (Heffron 1979). According to William Osler in the beginning of the century, pneumococcal pneumonia was "the most common and serious acute infection" (Osler 1901) and he named it "captain of the men of death".

In 1911 the first pneumococcal vaccine trials were started on South African gold miners, but were unsuccessful, partly due to inadequate knowledge of the many serologically distinct pneumococcal serotypes (Austrian 1981). During the thirties and forties new vaccines with increased efficacy were tested. At this time it was well known that leukocytes were capable to kill pneumococci and that anti-capsular antibodies accelerate phagocytosis induced by serum (Heffron 1979). From 1930 until recently, the name Diplococcus pneumoniae was used for these organisms.

The introduction of sulfonamides and later also antibiotics for treatment of pneumococcal infections, diminished for several years the interest of vaccination and the vaccines were withdrawn (Austrian 1981).

However, there is still a considerable morbidity in pneumococcal infections. The mortality, particularly among immunocompromised patients is high, even in patients treated with antibiotics (Austrian & Gold 1964, Mufson et al 1974). Furthermore, pneumococcal strains with multiple antibiotic resistance have been reported (Appelbaum et al 1977, Ward 1981). The interest in vaccination against Streptococcus pneumoniae, the current name of these organisms, has thus increased again.

S. pneumoniae (c f Austrian 1980). The pneumococcus is a gram-positive coccus, occurring in pair. When conditions of growth are good, the pneumococcus usually possesses a large capsule. The polysaccharides of the capsule are antigenic and form the basis of the classification of S. pneumoniae. At present more than 80 serologically defined pneumococcal capsular polysaccharide types are known, many of which are serologically related (Schiffman 1981). The Danish system of nomenclature for pneumococci combines closely related types into groups. Types within each group are given the same group number followed by different letters (Lund & Henriksen 1978). The four types of group 19 are for example entitled 19F, 19A, 19B, and 19C.

Components of the pneumococcal cell wall, i.e. the C-polysaccharide, which contains teichoic acid, are also of interest. The C-polysaccharide has capacity to interact with C-reactive protein (CRP) (Kaplan & Volankis 1974) and teichoic acid may activate the complement system (Winkelstein & Tomasz 1978).

The mechanisms responsible for the symptoms in pneumococcal disease are rather unknown (Austrian 1981, Johnston 1981). Also is unknown why some pneumococcal serotypes now are isolated more seldom in infections than in the thirties and the forties, and other serotypes now are found more frequently (White 1979, Austrian & Gold 1964, Mufson et al 1974). The polysaccharide capsule is essential for the virulence of the organism, but

the capsule itself lacks toxicity (Austrian 1981). Why some pneumococcal serotypes commonly cause infections while other types seldom or never are found in disease is not clarified.

It is possible that both the composition and the quantity of capsular polysaccharide play a role in virulence. Types 3 and 37 produce the largest capsules of any pneumococcal type (Austrian 1981). Serotype 3 is commonly found in infections and is in bacteremic pneumonias associated with very high mortality, whereas serotype 37 is almost without virulence. Serotype 12, which has a small capsule may be highly virulent. It has been shown that different virulence within the same serogroup of S. pneumoniae is related to the amount of their capsular polysaccharide (Austrian 1981).

A convincing role of pneumococcal toxins for the pathogenicity of the disease has not been obtained (Austrian 1980, Johnston 1981). It has been suggested that at least some part of pneumococcal disease may result from interactions between antibody, complement and phagocytic cells (Johnston 1981).

Recent surveys have found that about 70-80 % of pneumococcal infections are caused by 14 pneumococcal serotypes (Austrian 1976, Austrian 1981). Polysaccharides from these 14 serotypes are included in a current vaccine.

Current clinical importance of pneumococci. The introduction of antibiotics has reduced the seriousness of pneumococcal disease. It is obvious, however, that the widespread use of antibiotics has not eliminated the pneumococci as a common cause of morbidity and mortality.

S. pneumoniae is a major respiratory pathogen. Pneumococci are the most frequent bacterial organisms in acute otitis media, pneumonia and one of the most commonly isolated in sinusitis acuta (Roberts 1979).

Studies from Scandinavia indicate that about 25 % of all hospitalized pneumonias are caused by S. pneumoniae (Schreiner et al 1973). It has been estimated that the attack rate of pneumococcal pneumonia in USA is in the range of 1-10 per 1000 man-years (Austrian 1981). Certain population groups have increased frequency of pneumococcal infections, i.e. South African gold miners with about 100-200 cases yearly per 1000 individuals (Austrian 1976, Roberts 1979). The incidence of bacteremia during pneumococcal pneumonia has been calculated to 25-50 per 100 000 individuals (Austrian 1981). The mortality in bacteremic pneumococcal pneumonia was about 20-30 % in USA during the sixties (Austrian & Gold 1964, Mufson et al 1974, Roberts 1979).

Austrian and Gold (1964) found in a study of about 500 patients with bacteremic pneumococcal pneumonia treated with antibiotics, that most fatalities occurred within the fifth day of illness. In fact, it was only after

the fifth day that treatment with antibiotics was superior to the results earlier found with symptomatic treatment or serum therapy.

The incidence of pneumococcal meningitis in USA has been found to be approximately 1.5 per 100 000 (Austrian 1981). This figure is similar to recent estimates of the number of meningitis caused by pneumococci in Sweden (Jonsson & Alvin 1971, Alestig et al 1976). The mortality is still high, about 10-30 % in spite of treatment with antibiotics (Jonsson & Alvin 1971, Center for Disease Control 1979).

Increased susceptibility to pneumococcal infections is encountered in certain groups of patients. Patients with primary hypogammaglobulinemia or with secondary immunoglobulin deficiency due to diseases as myeloma, chronic lymphatic leukemia and lymphoma or treatment with immunosuppressive drugs or radiation show increased number of serious pneumococcal infections (Schwartz 1982). After splenectomy and in patients with functional asplenia due to various diseases, the frequency of fulminant pneumococcal infections is also increased (Singer 1973). Effective antibiotic treatment is often made impossible by the rapid course of pneumococcal infections in these individuals.

Host defence against infections with *S. pneumoniae*

Local factors. Since the pneumococci usually invade an individual from the respiratory tract, the ability to colonize the respiratory mucosa might be one crucial factor for the initiation of an infection.

Encapsulated pneumococci are found in nasopharynx or in throat in about 30 % of healthy children and in lower frequency among adults (Hendley et al 1975, Klein 1981). The pneumococcal serotypes most often associated with diseases, are also the most frequently encountered serotypes in isolates from healthy children. It has been found, that *S. pneumoniae* in vitro adhere poorly to oropharyngeal cells and a perhaps confusing observation is that the bacterial capsule may interfere with adherence (Selinger & Reed 1979).

Increased adherence to epithelial cells by several microorganisms, including *S. pneumoniae* has been observed during viral infections in the respiratory tract (Fainstein & Musher 1980). It has been observed that minor respiratory infections are associated with increased carrier rates of pneumococci (Heffron 1979). These observations might be the explanation for the increased frequency of pneumococcal disease after viral infections. However, viral infections may also compromise the function of phagocytes (Larson & Blades 1976, Kleinerman et al 1975, Jacob et al 1980).

Phagocyte host defence. After penetration into the tissues or the blood stream S. pneumoniae encounters the systemic host defence. The interaction with serum proteins, such as immunoglobulins and complement factors, generates chemotactic factors, which attract the phagocytes (Wilkinson 1980). Furthermore, the serum proteins prepare, opsonize, the bacteria for subsequent phagocytosis and killing by granulocytes, monocytes and fixed macrophages (Winkelstein 1973). The phagocytes have surface receptors which recognize the Fc part of immunoglobulin G and the complement component C3b (Stossel 1974).

Granulocytes are considered to be primarily responsible for the phagocytic host defence against extracellular bacteria such as S. pneumoniae (Densen & Mandell 1980).

Blood-borne S. pneumoniae are normally cleared by the spleen and subsequently phagocytized by splenic macrophages (Wara 1981). Also macrophages in other tissues, i.e. the liver participate in the phagocytic defence against S. pneumoniae (Brown et al 1981, Frank et al 1981).

Defects of any of the components of the phagocytic defence may contribute to increased susceptibility to infections with S. pneumoniae.

Immunoglobulins. The capsular polysaccharides and cell wall structures, such as teichoic acid are the main immunogenic components of the pneumococcus (Baddiley 1968, Austrian 1980). The importance of specific anti-capsular antibodies in defence to S. pneumoniae is demonstrated by:

1. Frequent pneumococcal infections in patients with hypogammaglobulinemia (Rosen & Janeway 1966).
2. Therapeutic effect of specific pneumococcal antisera, when used in the early phase of infection (Heffron 1979, Austrian & Gold 1964).
3. Type specific, preventive effect by vaccine containing pneumococcal capsular polysaccharides in some groups of patients with high risk for pneumococcal infections (Austrian et al 1976, Smit et al 1977, Amman et al 1977).

Since the interaction between polysaccharide capsule and specific antibodies implies conditions for complement activation, the microorganism might be recognized by both the Fc-receptor and C3b-receptor of the phagocyte. Animal experiments have shown both IgG and IgM to accelerate i.v. clearance of S. pneumoniae, but only in the presence of complement (Brown et al 1982). IgM was on molecular basis more efficient than IgG. Giebink et al (1977) found in vitro granulocyte phagocytosis of S. pneumoniae serotypes 6, 18, 23 and 25 to be reduced in hypogammaglobulinemic serum.

The role of antibodies to cell wall components, i.e. C-polysaccharide,

in pneumococcal opsonization is less clear. It is possible that antibodies and C3b, located on the cell wall, are shielded by the capsule from the receptors of the phagocyte (Winkelstein et al 1980).

C-reactive protein (CRP). CRP is a protein normally present in serum in low concentrations. CRP complexed with pneumococcal cell wall C-polysaccharide, can in the absence of immunoglobulins, activate C1 of the classical complement pathway (Kaplan & Volankis 1974). Since CRP is increasing rapidly during the first days of a bacterial infection, it might, in a non-immune individual, provide a mechanism for the activation of complement with subsequent S. pneumoniae phagocytosis. In mice, Mold et al (1981) have shown protective effect by CRP against i.v. injected pneumococci serotypes 3 and 4. Whether CRP serves a protective role against infections with S. pneumoniae also in humans is not known (Winkelstein 1981).

Tuftsins. Tuftsins are tetrapeptides with a proposed stimulating effect on phagocytosis. Reduced concentrations of tuftsins in serum have been reported in splenectomized individuals. The biological role of tuftsins is incompletely defined (Wara 1981).

fibronectin (c f Mosesson & Amrani 1980, Proctor et al 1982). Fibronectin is a glukoprotein produced by many types of cells. The protein has affinity to several structures, including cell surfaces. Fibronectin appears to mediate attachment of certain particles to macrophages.

Little is known of the interaction between fibronectin and microorganisms. fibronectin has been found to promote adhesion of granulocytes to some bacteria, i.e. S. aureus and S. pyogenes. Attachment of E. coli to granulocytes has not been found increased by fibronectin. These observations correlates with binding of fibronectin to S. aureus, but not to E. coli. However, adhesion of bacteria to granulocytes, mediated by fibronectin has not been associated with phagocytosis or killing.

The importance of fibronectin to host interaction with S. pneumoniae is largely unknown.

The complement system (c f Lachman 1979, Fearon & Austen 1980).

Complement (C)¹ is an enzyme system that mediates inflammatory reactions and plays an important role in resistance to a variety of microorganisms

¹ The nomenclature used convenes to the recommendations given in Bulletin of the World Health Organization 39:935 (1968) for components of the classical, and in Bulletin of the World Health Organization 59:489 (1981) for components of the alternative pathway of the complement system.

(Fine, 1981). C3 in its activated form, C3b, is the principal opsonin of the system. The role of C3 in defence against S. pneumoniae is well established (Winkelstein, 1981).

C3b can be generated through the classical (C1, C4, C2) or the alternative (factor B, factor D, properdin) pathway of complement activation. The terminal sequence C5, C6, C7, C8 and C9 (membrane attack complex) is common to the two pathways. Activation of the classical pathway is regulated by C1 inactivator, factor I and C4 binding protein. The factors I and H are regulators of the alternative pathway (Fig. 1).

Surface fixed or soluble immune complexes containing specific IgM and IgG antibodies bind to C1 through the interaction of Fc with the C1q subcomponent of the C1 complex (C1q, C1r, C1s). In the presence of Ca^{2+} the zymogens C1r and C1s are activated. C1s in its activated form cleaves C4 and C2 which in the presence of Mg^{2+} results in formation of the classical pathway C3 convertase (C4b2a). C-reactive protein in complex with certain substances, including pneumococcal C-polysaccharide, can also activate the classical pathway (Kaplan & Volankis 1974).

The alternative pathway is a positive feed-back mechanism for the activation of C3. Thus, the alternative pathway can amplify the generation of C3b following activation of C3 through the classical pathway. The alternative pathway can also be activated independent of the classical pathway.

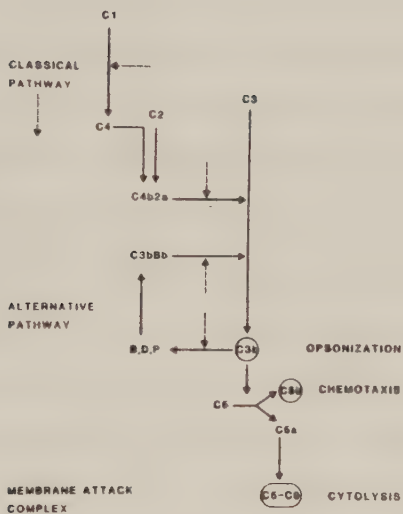


Figure 1 Schematic presentation of the complement system
Inhibitors are indicated by arrows with broken lines

According to recent evidence, C3b like molecules are continuously generated in serum by spontaneous hydrolysis of a thiolester bound in native C3 (Pangburn & Müller-Eberhard 1980). The C3b like molecule interact with other components of the alternative pathway and is thought to serve as a nucleus for alternative pathway C3 convertase (C3b,Bb). The enzyme, dependent on Mg^{2+} for its generation, is stabilized by P and inactivated by the factors I and H. C3b formed through the action of C3 convertase can bind to surfaces or substances that protect C3b and C3b-containing enzymes from the factors I and H. Such binding results in activation of the alternative pathway. It is known that sialic acid of certain surfaces increases the affinity of the inactivators to C3b. Capsular sialic acid is found only in some pathogenic organisms. Known activators of the alternative pathway include polysaccharides such as teichoic acid and lipopolysaccharides. The role of immunoglobulins in activation of the alternative pathway is not altogether clear (Winkelstein 1981, Gardner et al 1982).

Several biological functions are attributed to the complement system. In defence against bacterial infections, opsonization, which is dependent on fixation of C3b to the bacterial surface, is probably most important. Some bacteria are susceptible to the membranolytic effect of the terminal C5 to C9 sequence. The strong chemotactic activity for granulocytes and monocytes of C5 derived peptides may also have a considerable role in host defence.

Complement deficiency states (c f Agnello 1978). Selective deficiencies, inherited as autosomal recessive traits, have been described for most of the components of the complement system. Homozygous deficiencies of C1q, C1r, C1s, C4 or C2 appear to predispose for development of immune complex diseases such as SLE. Defects in the classical pathway are not clearly associated with susceptibility to bacterial infections. In contrast, C3 deficiency syndromes are characterized by repeated severe infections with a variety of bacteria. Deficiencies of C5, C6, C7 or C8 are associated with repeated disseminated infections with N. meningitidis or N. gonorrhoeae. The partial deficiency of C1 inactivator in hereditary angio-edema leads to a C4 and C2 deficiency syndrome, sometimes associated with SLE. Deficiencies of factor I or H give secondary C3 deficiency syndromes with increased susceptibility to bacterial infections.

Complement and S. pneumoniae. Pneumococci can activate both the classical and the alternative pathway of complement (Winkelstein 1981). The role of complement in defence against various serotypes of S. pneumoniae has been established in opsonophagocytic assays (Root et al 1972, Giebink et al 1977) and in animal experiments (Hosea et al 1980). Opsonization has been related to binding of C3, probably as C3b, to the organisms (Verbrugh et al 1979).

The classical pathway is initiated through interaction between the capsular polysaccharides and type-specific anticapsular antibodies (Johnston et al 1969) or by interaction of C-polysaccharide, CRP and C1 (Kaplan & Volankis 1974). Many pneumococci have capacity to bind C1q in the absence of antibodies or CRP (Prellner 1980). Teichoic acid in the cell wall, but not capsular polysaccharides seems to be responsible for this C1q binding. Activation of C2, however, requires antibody (Prellner 1981). The main role of anticapsular antibodies in opsonization of S. pneumoniae is probably to mediate fixation of C3 to the pneumococcal surface through classical pathway activation.

However, several pneumococcal capsular polysaccharides do not activate the alternative pathway, and other pneumococcal polysaccharides have this capacity only in high concentrations (Winkelstein et al 1976). Activation of the alternative pathway by pneumococcal cell wall structures, i.e. teichoic acid has been shown (Winkelstein & Tomasz 1977, Prellner 1981). Experiments by Winkelstein et al (1980) suggest that most C3b fixed through the alternative pathway by encapsulated pneumococci are bound to the cell wall, rather than to the capsule. According to recent evidence, C3b bound to cell walls of encapsulated S. pneumoniae are not opsonically active (Brown et al 1982).

With this background, the mechanism by which some pneumococcal serotypes are opsonized by the alternative pathway is not clear. Naturally occurring antibodies may enhance the ability of S. pneumoniae to activate the alter-

native pathway (Winkelstein et al 1972). This activity appears to reside in the $F(ab')_2$ part of the molecule and the specificity is probably directed towards a cross-reacting antigen present in other bacteria (Winkelstein & Shin 1974).

Granulocytes. Granulocyte accumulation is a characteristic feature of infections with S. pneumoniae, i.e. pneumonia (Heffron 1979). A major attractant of phagocytes, the complement fragment C5a, is formed by interaction of microorganisms with the complement system (Wilkinson 1980). The chemotaxis of the granulocytes, is mediated by the binding of C5a to a distinct receptor on the granulocyte (Snyderman & Goetzl 1981). Granulocytes also have a specific receptor for bacterial peptides.

The virulence of S. pneumoniae has been ascribed the anti-phagocytic properties of the capsule (Austrian 1980), which may be overcome by serum opsonins. Wood et al (1946) described granulocyte phagocytosis of pneumococci in the absence of opsonins, in conditions where the phagocyte appeared to squeeze the microorganism against other phagocytes or membranes in the body. However, this observation has not been verified in a recent study (Guckian et al 1980).

The interaction between opsonic immunoglobulins and C3b on the bacteria,

and the phagocyte receptors initiate the movement of the phagocyte membrane around the organism, until it is fully enclosed within a phagocytic vacuole (Griffin et al 1975) (Fig. 2). The cytoplasmatic granules fuse with the vacuole and discharge their contents in the proximity of the engulfed bacteria (Root & Cohen 1981). The ingestion phase is dependent on energy derived principally from glycolysis and therefore ingestion is efficient in anaerobic conditions (Sbarra & Karnovsky 1959). However, killing of microorganisms is partly dependent on oxygen.

Phagocytosis is associated with an increased consumption of oxygen, followed by production of oxygen metabolites with antibacterial effects, i. e. hydrogen peroxide (H_2O_2), superoxide (O_2^-), hydroxyl radicals (OH^-) and singlet oxygen (1O_2) (Root & Cohen 1981). H_2O_2 formed during granulocyte phagocytosis constitute together with the granular protein myeloperoxidase (MPO) and a halide, a potent intragranulocytic microbicidal system (Klebanoff 1967). In chronic granulomatous disease (CGD), granulocyte production of H_2O_2 is deficient (Holmes et al 1967). However, S. pneumoniae are easily killed by granulocytes from patients with CGD. H_2O_2 produced by S. pneumoniae might participate in MPO- H_2O_2 -mediated S. pneumoniae killing in these granulocytes, since H_2O_2 -deficient mutants of S. pneumoniae are not killed by granulocytes from CGD patients (Pitt & Bernheimer 1974).

Besides MPO, granules in the granulocyte contain several elements with

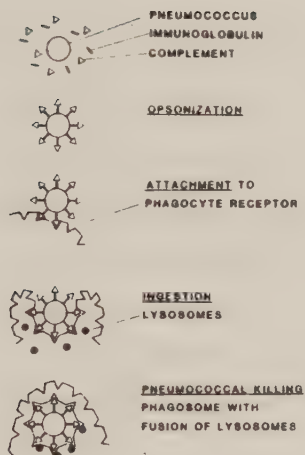


Figure 2 Opsonization and phagocytic killing
of *S. pneumoniae*

in vitro antibacterial activity (Root & Cohen 1981). A group of these substances, cationic chymotrypsin like proteins might participate in granulocytic S. pneumoniae killing, since they have a potent bactericidal effect on gram-positive organisms (Odeberg & Olsson 1975).

Monocytes-macrophages (c f Densen & Mandell 1980). Mononuclear phagocytes, circulating monocytes or tissue-bound macrophages which emanate from the circulating monocytes, appear important particularly to intracellular pathogens. They are rather long lived cells and the functional properties vary and depend on degree of activation, but also on the location in the body. The mononuclear phagocytes have receptors for C3b and for the Fc portion of IgG and they have both oxygen-dependent and oxygen-independent mechanisms for bacterial killing.

Alveolar macrophages serve as the first line of defence against microorganisms invading the lungs. Both immunoglobulins and complement seem necessary for efficient phagocytosis of pneumococci by alveolar macrophages (Guckian et al 1980). The interaction between pneumococci and the macrophages in liver and spleen will be discussed in a later section.

The spleen. The asplenic patient has a well established susceptibility to severe bacterial infections (Singer 1973). About 50 % of the septicemias

in splenectomized individuals are caused by S. pneumoniae. The mortality of septicemia in asplenic patients is around 50 % (Singer 1973, Wara 1981). The frequency of postsplenectomy sepsis differs and is largest among patients with other complicating diseases, i.e. Hodgkin's disease.

The role of spleen in host defence to pneumococcal infections is complex. Both qualitative and quantitative changes among opsonins and phagocytes are described (Wara 1981).

The unique anatomy of the spleen allows for prolonged contact between the splenic phagocytes and the blood-borne particles and presumably allows phagocytosis of poorly opsonized organisms (Ellis & Smith 1966, Wara 1981). Retarded clearance of pneumococci and other particles in splenectomized animals could be overcome by increasing the amount of IgG antibodies (Hosea et al 1981a), probably by an increased hepatic uptake. An intact function of the complement system also seems to be necessary for normal hepatic clearance of pneumococci. In the absence of a normal spleen there seems to be an increased requirement for opsonization, for pneumococcal phagocytosis by other parts of the reticuloendothelial system (Schulkind et al 1967, Hosea et al 1981 a).

Most studies have shown reduced levels of IgM in splenectomized individuals (Krivit 1977). Serum concentration of IgM after pneumococcal vaccination has been found both normal (Wara 1981) and reduced (Hosea

et al 1981b). In animals the spleen appears to play a major role in the immune response to soluble pneumococcal polysaccharide antigens (Amstrong et al 1978, Havas et al 1979). I.v. injection of killed pneumococci results in antibody production within a few hours and this response does not occur if the spleen is removed (Ellis & Smith 1966). Subcutaneous administration of pneumococcal vaccine to splenectomized individuals has been found to result in a rather normal or moderately reduced antibody response (Amman et al 1977, Sullivan et al 1978, Giebink et al 1980, Hosea et al 1981b). The way to present the antigen seems thus decisive for the magnitude of response in splenectomized individuals.

Evidence for defective function of the alternative complement pathway in some asplenic individuals has been presented (Corry et al 1979). However, the deficiency does not appear common (Graffner et al 1982) and pneumococcal in vitro opsonization is not substantially influenced (Winkelstein et al 1975).

The role of T cells in resistance to encapsulated organisms is rather unknown, but one function is to regulate the antibody synthesis in B cells (cf Käyhty 1981). The polysaccharide antigens have been called helper T cell independent antigens. However, in blood from mice suppressor and amplifier T cells have been found to have a regulatory effect on the response to pneumococcal capsular polysaccharide.

Removal of the spleen may disturb T-B cell cooperation (Amsbaugh et al 1978, Rozing et al 1978). Increased number of both B and T lymphocytes has recently been shown in peripheral blood from splenectomized individuals (Graffner et al 1982) and the subset of T cells with supposed suppressor function was found increased. However, methodological difficulties to distinguish between various types of T cells may have consequences for this observation (Editorial Lancet 1980).

Pneumococcal vaccination. Pneumococcal vaccines, based on capsular polysaccharides of the most common pneumococcal serotypes found in serious infections, have been developed. They have been widely used and after administration of several million doses, only few and poorly documented, severe side effects have been reported (Schwartz 1982). Trials performed by Austrian et al (1976) have shown significant reduction of pneumococcal pneumonia and septicemia among South African gold miners. In another high risk group, i.e. patients with sickle cell disease, Amman et al (1977) found reduced pneumococcal bacteremia after vaccination.

The clinical effect in other high risk groups, i.e. patients immunocompromised due to hematological diseases or due to immunosuppressive therapy, has not yet been established. In populations with a rather normal incidence of serious pneumococcal infections, no significant

protection is achieved by pneumococcal vaccination (Schwartz 1982) and the protective effect to otitis media in children is restricted (Mäkelä et al 1980, Sloyer et al 1981).

The immunologic response to pneumococcal immunization has been widely studied during the last years. The antibody levels have been measured, mostly with a radioimmunoassay (RIA), which determines both low and high avidity antibodies (Callahan et al 1979). The correlation of antibody levels to clinical protection is of interest, but cannot be defined exactly at present.

THE PRESENT INVESTIGATION

Aims of the present investigation

The intention with the present study was to investigate the interaction between S. pneumoniae and various components of the host defence, i. e. granulocytes, immunoglobulins and complement components.

The following main aspects were studied:

1. The sensitivity of various serotypes of S. pneumoniae to granulocyte phagocytosis and killing was studied to evaluate the hypothesis, that clinical virulence of different serotypes would correlate with resistance to granulocytes.
2. Hereditary properdin deficiency was described. The deficiency was studied with particular reference to alternative complement pathway function and to pneumococcal opsonization.
3. Effect of pneumococcal vaccination in splenectomized and/or immuno-suppressed patients was studied with respect to production of opsonic antibodies.
4. Following pneumococcal vaccination, the frequency of cross-reacting, opsonic antibodies to clinically important, not vaccine included pneumococcal serotypes was determined.

Materials and methods

Granulocytes and monocytes. Granulocytes were isolated from peripheral blood of healthy individuals using the Isopaque-Ficoll technique with some modifications. More than 90 % of the cells were granulocytes. Mononuclear cells were harvested from the interphase of the Isopaque-Ficoll separation. The mononuclear cell suspension contained approximately 15-25 % monocytes.

Bacteria. The pneumococci were stored in foetal calf serum in -80°C and cultured on blood agar. The cultures were renewed monthly from the strains kept at -80°C . Before experiments the bacteria were cultured in an anaerobic environment for four hours in broth. After washings the log-phase organisms were diluted in buffer to about 5×10^7 viable pneumococci per ml.

The pneumococci used in our study were checked at several occasions for positive Quellung reaction. Experiments with serotype 14 and 23 pneumococci did not show significant difference in sensitivity to phagocytic killing, between pneumococci cultured as described and pneumococci which were passed through mice. It has been observed that repeated passage of pneumococci on solid media may result in changes in encapsulation and virulence. This decay of the pneumococcal virulence probably depends on conditions of cultivation. Thus, it has been found possible to retain

virulence for long periods with other media (White 1979, Lund & Henrichsen 1978).

Phagocytic killing of pneumococci. About 2×10^7 bacteria per ml were incubated with diluted serum and phagocytes during rotation end-over-end (I, IV) or during continuous, slow agitation for 30 minutes at 37°C . Granulocytes or monocytes were added to a concentration of approximately 2 or 4×10^6 per ml. Controls were run without granulocytes. Viable bacteria were quantified by colony counting with the pour plate method after lysis of phagocytes in distilled water.

Granulocyte-associated pneumococci. Viable granulocyte-associated bacteria were determined after removal of non-adherent pneumococci by differential centrifugation at $160 \times g$ at $+4^{\circ}\text{C}$. The number of colony-forming units in the granulocyte pellet was determined after granulocyte lysis.

Granulocyte chemotaxis. Chemotactic activity was determined according to Nelson et al (1975) as detailed in V.

Quantitation of pneumococcal antibodies. Specific anticapsular antibodies to serotypes 3, 6A, 14, 19F and 23F were determined with enzyme-linked immunosorbent assay (ELISA) (Karup Pedersen & Henrichsen 1982a).

Antibodies to S. pneumoniae serotypes 6B, 9N and 19A were measured with a protein-A binding assay as detailed in VI. In this assay the pneumococci were allowed to interact with diluted pre- or postvaccination sera for the binding of serum antibodies to the organisms. After washings ^{125}I -labeled staphylococcal protein A was added in excess the antibody-coated pneumococci. Following further incubation the radioactivity in the bacterial pellet was measured. The uptake of ^{125}I protein A was corrected for the number of bacteria and the protein-A binding in the absence of serum.

Serum and EDTA plasma. Samples of serum and plasma from patients and healthy controls were stored in aliquots at -80°C . In many experiments a pool of 20 normal sera was used as a reference. For inactivation of complement, sera were heated at 56°C for 30 minutes.

Complement studies. Complement components were quantitated immunochemically with electroimmunoassay and functional tests were carried out as described in II.

C3 binding to pneumococci. S. pneumoniae serotype 23F was incubated with diluted serum at 37°C . Commercial anti-C3d IgG labeled with ^{125}I was added. After further incubation, the bacteria were washed and the radioactivity in the bacterial pellet was measured as indicated in IV.

RESULTS

Opsonophagocytic killing of *S. pneumoniae* (I)

The susceptibility of 10 pneumococcal serotypes to killing by human granulocytes was evaluated in the presence of serum. Five serotypes (3, 6, 14, 19, 23) commonly associated with infections, "pathogenic", and five serotypes (31, 35, 36, 42, 43) rarely found in infections, "non-pathogenic", were studied.

Following incubation of bacteria and granulocytes in diluted pooled human serum, the number of surviving bacteria was determined by the pour plate method, as colony forming units. The number of cell-associated, viable pneumococci was ascertained after differential centrifugation. The fraction of granulocyte-associated, viable bacteria was low for all ten serotypes. In incubations with 4 % serum, the cell-associated bacteria accounted for $1.6 \% \pm 1.6 \%$ (mean and S.D.) of the total number of viable bacteria at 30 minutes. In 8 % serum, $5.4 \% \pm 4.9 \%$ of the surviving organisms were cell-associated. The number of intracellularly surviving pneumococci was thus low for all serotypes, indicating intra-granulocytic killing to be efficient.

We found a considerable variation in sensitivity to an in vitro MPO- H_2O_2 system among the five pathogenic and the five non-pathogenic

pneumococcal serotypes. No correlation between tendency to cause infection, and resistance to this bactericidal system was found. Similar results were observed with antibacterial cationic granular proteins.

Since intra-granulocytic killing of pneumococci was efficient and no extra-cellular killing occurred, we could use granulocyte killing as a measure of opsonization and phagocytosis of pneumococci. Granulocyte phagocytic killing of the five pathogenic and the five non-pathogenic serotypes in diluted control serum were compared. In 4 % serum three non-pathogenic serotypes (35, 42, 43) were killed by granulocytes. In 8 % serum both pathogenic (6, 14, 23) and non-pathogenic (35, 36, 42, 43) serotypes were killed. Two pathogenic (3, 19) and one non-pathogenic (31) serotypes were not substantially phagocytized at this serum concentration. Serotypes 19 and 31 were efficiently opsonized at 16 % serum, while serotype 3 was not killed even at high serum concentrations (80 %).

Antibodies, as measured with ELISA, to the five pathogenic serotypes were found normal in the pooled control serum used in these experiments. Since no conventional method for evaluation of antibodies to non-pathogenic serotypes has been developed, the antibody levels to these serotypes were studied with the protein-A binding assay. Using this method antibodies were found, in the pooled serum, to all non-pathogenic and pathogenic serotypes. The lowest value was noticed for one pathogenic

serotype (type 6).

Our results thus show no strict correlation between the tendency of S. pneumoniae to cause infections and the amount of serum necessary for granulocyte opsonization and phagocytosis.

Monocyte phagocytosis of three pathogenic (6, 19, 23) and two non-pathogenic (35, 43) pneumococcal serotypes in diluted, pooled serum were studied. The results were similar to those found with granulocytes. Serotype 43 was most sensitive to and 19 most resistant to monocyte phagocytic killing. The pathogenic serotype 23 and the non-pathogenic serotype 35 had equal requirement for opsonins.

Granulocyte phagocytosis of the five pathogenic and the five non-pathogenic pneumococcal serotypes were also compared in serum from a patient with C2 deficiency, in MgEGTA chelated pooled control serum and in serum from a patient with hypogammaglobulinemia. A complete inhibition of phagocytosis of all serotypes, except for type 35 was found in 8 % C2 deficient or MgEGTA chelated serum. In 16 % chelated serum, the phagocytosis of the tested serotypes, three pathogenic and one non-pathogenic, were similarly increased. The phagocytic killing in the hypogammaglobulinemia serum was, however, found mainly identical with that in pooled control serum. The immunoglobulin deficient serum was later tested for pneumococcal antibodies with ELISA and found to contain

rather normal levels of anti-capsular antibodies to pneumococci.

Discussion. A major problem, when studying phagocyte killing of bacteria, is the separation of viable, intra-cellular from viable, extra-cellular microorganisms. A number of methods have been developed for the study of intra-cellular survival of organisms, such as differential centrifugation, elimination of extra-cellular bacteria with the use of antibiotics or, for S. aureus the use of lysostaphin (Maalö 1946, Alexander et al 1968, Tan et al 1971, Solberg 1972, Koch 1974). Differential centrifugation is simple, but does not distinguish between phagocytized bacteria or organisms only adherent to the phagocytes. Since only a low ratio of the total number of viable pneumococci was found cell-associated, the method of differential centrifugation permitted the conclusion of efficient granulocyte killing of S. pneumoniae.

The information on resistance of pathogenic and non-pathogenic pneumococci to granulocyte phagocytosis is limited. Smith & Wood (1969) found an unencapsulated strain of serotype 36 to be easily phagocytized compared with a more virulent strain. Giebink et al (1977) studied extensively three common pathogenic serotypes (6, 18, 23) and one serotype rather seldom associated with infections (25). However, the serotype 25 was isolated from a patient with bacteremia and was as resistant to granulocytes as the other three serotypes.

Opsonic C3b, in non-immune individuals, may be provided by the alternative pathway of complement and it has been shown that pneumococci can activate the alternative pathway (Fine 1975, Stephens et al 1977, Prellner 1979). Opsonization of some pathogenic serotypes of S. pneumoniae mediated by the alternative pathway alone has been reported reduced, compared to opsonization in serum with intact classical and alternative pathways (Root et al 1972, Giebink et al 1977). Fine (1975) has suggested that varying pathogenicity among pneumococcal serotypes may be associated with different ability to activate the alternative pathway. However, Stephens et al (1977) found a great variability in alternative pathway activation by both pathogenic and non-pathogenic pneumococcal serotypes. Our results do not indicate alternative pathway to mediate better opsonization of non-pathogenic compared to pathogenic serotypes of S. pneumoniae.

The importance of the monocyte - macrophage system in human defence to pneumococcal infections is indicated by the increased frequency of overwhelming infections in splenectomized individuals (Singer 1973, Wara 1981). We found the capacity of monocytes to kill pneumococci very similar to that of granulocytes. The tissue-bound macrophages have their origin from the circulating monocytes (Volkman 1970). However, our observation of similar requirement of serum for monocyte phagocytic killing of pneumococci as for granulocyte killing, may not be relevant to all types of fixed macrophages. Animal experiments have shown that macrophages located in different tissues have variable need for opsonins

(Brown et al 1981). Intra-monocytic killing of bacteria may also differ due to variations in lysosomal bactericidal agents (Edelson 1982).

The aim of the study described in this paper, was to evaluate if the variable tendency of pneumococcal serotypes to cause infections could be determined by their resistance to phagocytic killing. This hypothesis was not supported by our study or the scanty information in the litterature. Other mechanisms might be essential for the different capacity of S. pneumoniae serotypes to cause disease.

In general a good correlation between serotypes found in nasopharynx or throat of healthy carriers, and serotypes associated with disease are found (Hendley et al 1975, Klein 1981). More than 80 % of pneumococcal isolates in nasopharynx or throat in healthy individuals are of serotypes commonly found in disease (Gray et al 1979, Klein 1981). Therefore, the ability of S. pneumoniae serotypes to colonize the nasopharynx might be decisive for pathogenicity. The adhesive capacity of pneumococci to epithelial cells in upper respiratory tract has been studied by Andersson et al (1981). He found a great variation in adhesive capacity within different strains of the same, pathogenic serotypes. In general, strains from patients with otitis media and from healthy carriers adhered in a higher degree, than did strains isolated from blood or spinal fluid. No relationship was found between the polysaccharide capsule type and adhesive capacity. Selinger and Reed (1979) noticed, that an increase in

pneumococcal encapsulation was related to a decrease in adherence to oropharyngeal cells. It is not known if non-capsular surface factors are of importance to the adhesion of pneumococci. It has been demonstrated for group A streptococci, that lipoteichoic acid and M-protein both promote adhesion (Ellen & Gibbons 1974, Peterson & Quie 1981).

Influence by deficiency of properdin on alternative complement pathway function and on pneumococcal opsonization (II, III and IV)

Family with properdin deficiency (II and III). Two young brothers and an uncle in a family died with several years interval in meningococcal infections. Another member of the kindred, a 6-year old boy died rapidly with symptoms of an infectious disease. The diseases were characterized by a fulminant course. No general tendency to infections or other diseases was observed among family members.

Sera from one of the brothers (the index patient) who died in a meningococcal infection and from two other males, healthy when studied, showed a complete lack of the alternative complement pathway component properdin (P). Slightly reduced P levels were found in three female members of the family. The parents to the deceased patients showed normal P levels. Other components of the complement system and serum proteins as CRP and immunoglobulins were normal or modestly increased, with

exception of indications of complement activation in the boy who died. Granulocyte phagocytic capacity, lymphocyte surface markers, lymphocyte stimulation with PHA, skin testing, and antibody response to immunization appeared normal.

Complement functions in P deficient serum (II and III). Impaired alternative complement pathway functions were demonstrated. The P deficient sera gave "normal or moderately increased values in the CH 50 test. Screening assays for alternative and classical complement pathways by hemolysis in gel showed in the P deficient sera only partial lysis for the alternative pathway, while the pattern was normal in the test for the classical pathway. Low background conversion of C3 was found in P deficient sera. Restitution with P at physiological concentration normalized background C3 cleavage and hemolysis in gel. Incubation with inulin or zymosan resulted in low C3 cleavage unless purified P was added. The uptake by normal granulocytes of endotoxin-coated oil particles was reduced in P deficient serum. Addition of purified P also in subnormal concentrations resulted in opsonization of the particles in an almost normal level. Stossel et al (1973) had suggested the measurement of opsonization of such particles to comprise a functional assay for the alternative complement pathway.

Other complement mediated activities were also found reduced in P deficient serum. A moderately reduced serum bactericidal efficiency to

a group C meningococci was noticed. We also found reduced generation of granulocyte chemotactic activity by P deficient serum and pneumococci (C. Rosén & J.H. Braconier, Generation of granulocyte chemotactic activity by virulent and avirulent serotypes of S. pneumoniae, to be published).

Pneumococcal opsonization (IV). Opsonization of S. pneumoniae in serum with deficiency of P was evaluated. Phagocytic killing of five pneumococcal serotypes (6A, 14, 19F, 23F and 35^b) was found reduced in diluted P deficient serum as compared with diluted control serum. The following data suggested that the P deficiency alone accounted for the insufficient opsonization:

1. Normal levels of specific antibodies to capsule polysaccharide types 6A, 14, 19F and 23F, as determined by ELISA, in the P deficient serum.
2. Increased opsonization of serotype 23F after addition of fresh, but not of heat-inactivated serum to the P deficient serum.
3. Restored opsonization of serotype 23F by addition of purified P in concentrations from 20 to 100 % of normal.
4. Purified P restores the reduced C3-binding to pneumococcal surface structures, found after incubation in P deficient serum.

Opsonization of S. pneumoniae serotype 23F in MgEGTA chelated control serum was much reduced compared with opsonization provided by unche-

lated control serum, which indicates insufficient opsonic activity by the alternative complement pathway. In unchelated P deficient serum, also a reduced opsonization was found. These observations suggest a reduced function of the C3 amplification in P deficient serum.

These findings were supported by unpublished experiments measuring C3-fixation to serotype 23F pneumococci. The bacteria were preincubated with 8 % control serum and following washings, chelated control or P deficient sera at 16 % were added. It was found, that binding of anti-C3d rapidly was increased by addition of chelated control serum, in contrast to the only minor increase found in experiments with chelated P deficient serum. The C3-binding to non-preopsonized pneumococci was low in both types of chelated sera. A positive correlation between opsonization and the amount of surface bound C3 has earlier been found for some bacteria (Verbrugh et al 1979).

Opsonization of serotype 23F pneumococci in P deficient serum was not enhanced by addition of heat-inactivated control serum, suggesting P to be heat labile. This hypothesis was further tested. It was found that heat-treated, purified P, neither restored opsonization of pneumococci in P deficient serum, nor enhanced C3 activation in the same serum when incubated with zymozan.

Discussion. Deficiency of P was demonstrated as defined by immunochemi-

cal and functional criteria. This deficiency might have predisposed for the meningococcal infections in the family and in contrast to other complement deficiency states (Agnello 1978) it appeared to be X-linked.

The only established function of P, is to stabilize the alternative pathway C3 convertase, C3b,Bb (Fearon & Austen 1980). By the amplification loop, C3b, generated by the alternative or classical pathways, may provide more C3b,Bb for further cleavage of C3, resulting in increased amounts of opsonically active C3b. Thus, deficiency of P would be likely to limit the amount of C3b generated through both the classical and alternative pathways, as was indicated by the experiments in this study with S. pneumoniae serotype 23F.

The opsonic capacity of P deficient serum was reduced, as shown with both five S. pneumoniae serotypes and with endotoxin-coated oil particles. The restored opsonic activity in P deficient serum to serotype 23F pneumococci after addition of rather low amounts of purified P, is in agreement with the normal opsonization of pneumococci found in serum from patients with partial P deficiency (Davis & Forristal 1980)

Reduced formation of C3b will also, besides the effect on opsonization, affect other functions of the complement system. Complement mediated cell-lysis is of importance in defence against several types of microbes, i.e. gram-negative bacteria (Editorial, Ann Intern Med 1979,

Fine 1981). Recurrent neisserial infections are described among patients with deficiencies of complement components C5, C6, C7 or C8 (Petersen et al 1979). The less fulminant course of the meningococcal infections in these patients, compared with the individuals with P deficiency, might be explained by the preserved opsonic capacity in sera with defects of the late complement components (Matthews et al 1980).

Our results indicate P to be heat labile. It has been reported that activated P retained its activity in a hemolytic assay after heat treatment (Minta & Vetvutanapibul 1978). P exists in at least two forms (Götze et al 1977) and it is possible that the activated P used by Minta & Vetvutanapibul and the native P used by us differs in susceptibility to heat treatment.

There was no indication of sensitivity to pneumococcal infections in the family with P deficiency. Isolated deficiencies in the classical complement pathway, i.e. lack of C4 and C2 are usually not associated with pneumococcal disease (Agnello 1978). Increased number of pneumococcal and other bacterial infections are seen in patients with C3 deficiency syndroms (Agnello 1978) and in the two patients with C2 deficiency combined with low levels of factor B of the alternative pathway (Newman et al 1978). Pneumococcal infections are thus more often seen in patients with deficiency of C3, but not in patients with intact function of either the alternative or the classical

pathway. The increased sensitivity to infections in patients with hypogammaglobulinemia is not conflicting with this concept. Evidences are accumulating that immunoglobulins influence the activity of the alternative as well as the classical complement pathway interaction with pneumococci (Edwards et al 1980, Gardner et al 1982).

Opsonic antibodies in immunocompromised patients after immunization with a 14-valent pneumococcal vaccine (V and VI)

A. Effect on some serotypes included in the pneumococcal vaccine (V).

S. pneumoniae serotypes 6A, 19F and 23F were studied due to their proposed, different capacity to induce specific antibody increase after vaccination (Hilleman et al 1981). Antibody (measured with ELISA) and opsonic response to immunization were evaluated in eight patients with malignant diseases and in 23 patients splenectomized due to trauma or various malignant or non-malignant diseases.

About 50 % of the opsonic assays showed increased opsonic capacity in postvaccination sera. At least twofold antibody rise to these serotypes was found in the same frequency after vaccination. Close relationship between the results of opsonic and antibody assays was found. Sera with antibody increase more than 150 arbitrary units

showed enhanced opsonization in 93 % (43/46) of the assays. Only 13 % (6/48) of sera with antibody rise less than 100 units were associated with increased opsonization of pneumococci.

No difference in the response to immunization among patients with low (≤ 50 units) or high (≥ 200 units) prevaccination antibody concentration was found. Postvaccination sera showed, in the different groups of patients, enhanced opsonic activity in 46-63 % of the assays and at least twofold antibody rise in 50-63 %. These variations between the groups were not significant. Five patients with Hodgkin's disease were vaccinated and studied during chemotherapy and two begun chemotherapy soon after immunization, before the postvaccination serum samples were drawn. Both the numbers of enhanced opsonic assays (5/21) and the numbers of increased antibody concentrations (7/21) were significantly lower than among the remainder of the patients.

In patients splenectomized less than a year before vaccination, both twofold antibody rise and enhanced opsonic activity were seen significantly more often than in individuals with a longer interval between immunization and splenectomy. Individuals older than 60 years showed positive response to vaccination as often as younger individuals. The three serotypes, 6A, 19F and 23F, were found equally efficient to induce significant postvaccination rise in opsonization or antibody levels.

Discussion. At least 80 % of healthy individuals above two years of age have twofold or greater increase in antibodies (RIA) to all vaccine serotypes (Hilleman 1981). Our study did not include a control material. However, with the ELISA used, Karup Pedersen et al (1982 b) have found, that about 60 % of the assays in healthy individuals showed at least twofold antibody increases after vaccination. This indicates that the patients in our study displayed a twofold or greater antibody increase in about the same frequency as found in normal individuals. Other studies have also shown normal or moderately reduced antibody response among splenectomized individuals after pneumococcal vaccination (Amman et al 1977, Sullivan et al 1978, Giebink et al 1980, Hosea et al 1981 b). However, no accepted standard of adequate antibody response exists (Siber et al 1980 a, Schwartz 1982). Twofold or greater increase in postvaccination antibody level is often used as an index of a positive response, but sometimes 1.4-fold increase is used. Neither of these levels is necessarily correlated with protection against disease. A twofold increase may not be sufficient if preimmunization antibody concentrations are low.

Other reports on enhancement of opsonic activity after pneumococcal vaccination have shown inferior relationship to antibody increase (Giebink et al 1980, Simberkoff et al 1980, Johansen & Karup Pedersen 1982). The discrepancies between these studies and ours might depend on methodological conditions. Serotype 3, which has a very prominent capsule

(Austrian 1981), and is rather resistant to opsonophagocytic killing (Austrian & Gold 1964, Mufson et al 1974, 1), has been the test organism in some studies (Simberkoff et al 1980, Johansen & Karup Pedersen 1982). The sensitivity of the opsonic tests was low, due to the use of only one serum concentration (Giebink et al 1980) or due to a high requirement for significant opsonic increase (Simberkoff et al 1980).

The similar immunogenicity of polysaccharide types 6A, 19F and 23F and the influence of interval between splenectomy and vaccination found in this study, is contrasting to other reports (Giebink et al 1980, Hilleman et al 1981). The reason for these differences is unclear. The large individual variation in response to polysaccharide antigens (F. Karup Pedersen: Antibody response to pneumococcal vaccine in splenectomized children, to be published) and the rather small groups of patients studied may be one explanation.

The reduced antibody response after pneumococcal vaccination in patients with immunosuppressive treatment for Hodgkin's disease is in agreement with other studies (Siber et al 1978, Minor et al 1979, Amman et al 1981). Thus, among the patients with the highest risk for severe pneumococcal infection, the vaccination seems to provide a rather low degree of protection.

B. Effect of pneumococcal vaccination on clinically important serotypes, not included in the vaccine (VI)

Serotypes 6B, 9V, and 19A are of clinical interest since they, according to a recent study (Broome et al 1980), caused about 14 % of serious pneumococcal infections. Furthermore, some strains of S. pneumoniae serotypes 6B and 19A have shown multiple antibiotic resistance (Appelbaum et al 1977, Radetsky et al 1981). Thus, the degree of cross-protection provided by vaccination with the serologically related serotypes 6A, 9N, and 19F, included in the current vaccine, was considered of great interest.

Opsonic activity to pneumococcal serotypes 6B, 9V, and 19A was studied. Pre- and postvaccination sera from patients, who had responded to vaccination with enhanced opsonization and at least twofold antibody increase to the vaccine polysaccharides 6A, 19F and 23F as measured with ELISA, were selected for the study. Furthermore, antibody levels to serotypes 6B, 9V, and 19A were determined with a protein-A binding assay.

Enhanced opsonic activity to the serotypes 6B, 9V, and 19A was found in 8, 4, and 7 patients respectively. A twofold or greater increase in antibody levels in postvaccination sera to the same pneumococci was found in 10, 3, and 7 of the patients respectively. A close agreement between increased antibody levels and enhanced opsonization was found.

Discussion. Cross-reacting opsonic antibodies to the serotypes 6B and 19A, not included in the vaccine were thus found after pneumococcal vaccination in most patients. Anticapsular IgG antibodies are found in the IgG2 sub-class (Riesen et al 1976, Siber et al 1980b) and the staphylococcal protein A has an anti-immunoglobulin specificity for IgG1, IgG2, IgG4 and for some IgA and IgM antibodies (Kronvall & Williams 1969, Harboe & Fölling 1974). It has been claimed that binding of complement components to antigen-antibody complexes could interfere with protein-A binding (Scharfstein et al 1979). Since our experiments were performed under conditions which permitted activation of complement, control experiments to evaluate the influence of heat-inactivation of serum on protein-A binding were done. However, studies with sera from patients showed only about 10 % higher protein-A binding in heat-inactivated than in non-inactivated sera.

Extensive cross-reactivity between serotypes 6A and 6B, as measured with RIA, has been noticed by Robbins et al (1979) among healthy individuals. Lee et al (1981) found that 80 % (12/15) of volunteers vaccinated with 19F polysaccharide, had at least twofold antibody increase (RIA) to the homologous serotype and only 20 % showed a similar rise to serotype 19A. The selection of only sera with high antibody response to vaccine type 19F, as assessed with ELISA, might partly explain the higher degree of cross-reactivity between serotypes 19A and 19F in our study. We found only weak cross-reactivity between 9N and 9V poly-

saccharides. Szu et al (1982) observed that, after immunization with polysaccharide type 9V or 9N only about 50 % of volunteers showed at least twofold increase in antibody concentration to the heterologous type, as measured with RIA. Twofold increase to the homologous type was more often found. These results indicate that pneumococcal vaccine may induce opsonic antibodies to some clinically important serotypes not included in the vaccine. The protection provided by the present group 9 vaccine polysaccharide, 9N, to serotype 9V, the most common group 9 organism isolated among children (Austrian 1979), appears to be insufficient and may indicate a change in the vaccine composition.

The introduction of immunoprophylaxis with pneumococcal vaccines underscores the necessity for monitoring pneumococcal serotypes associated with infection. Such typing will detect changes in order of frequency which might influence vaccine composition and also give information of the clinical efficiency of the different polysaccharides included in the vaccine.

SUMMARY

Susceptibility to granulocyte killing of five commonly and five more rarely pathogenic serotypes of S. pneumoniae were studied. Intact granulocytes showed efficient killing of all serotypes. No relationship between resistance to certain intra-granulocytic microbicidal systems and the tendency to cause infections was found.

Granulocyte phagocytosis of the different pneumococcal serotypes was dependent on the concentration of serum opsonins, but again no strict relationship between opsonic requirement and pathogenicity was established. Similar results were found with monocytes. In conclusion, other mechanisms than resistance to opsonophagocytic killing by phagocytes must be important to the virulence of S. pneumoniae.

An inherited, selective deficiency of properdin (P), a component of the alternative pathway of complement, was described among male members of a family with high incidence of fulminant meningococcal infections.

Several alternative pathway functions were impaired in P deficient serum, while the classical pathway of complement appeared to be intact. The bactericidal effect of P deficient serum on N. meningitidis was moderately reduced. Opsonization of pneumococci and other par-

ticles in P deficient serum were clearly insufficient, as well as the binding of C3 to S. pneumoniae. Restitution with purified P restored the functional defects. Findings suggested that the activity of native properdin is heat labile (56°C, 30 minutes).

Opsonic antibody responses to immunization with a 14-valent pneumococcal vaccine were studied in 23 patients splenectomized due to various diseases and in eight patients with malignant diseases. It was found that approximately 50 % of the vaccinated patients, after immunization showed significantly enhanced opsonic activity and a twofold or greater rise in antibody levels, as measured by ELISA. After vaccination, a close relationship was found between enhancement of opsonization and at least twofold increase in antibody concentration.

The immunogenicity of the tested serotypes (6A, 19F, 23F) were similar. The response to vaccination among patients with present treatment for Hodgkin's disease was reduced. In conclusion, antibodies produced in response to pneumococcal vaccination were shown to be opsonically active. However, profoundly immunosuppressed patients, who are at high risk for severe pneumococcal infections have a reduced response to vaccination.

Cross-reacting opsonic antibodies to three clinically important, non-

-vaccine included serotypes (6B, 9V and 19A) were evaluated in sera from 10, selected patients who were vaccinated with the 14-valent vaccine. Enhanced opsonic activity to each of the S. pneumoniae serotypes 6B, 9V and 19A was found in 8, 4 and 7 patients, respectively. A twofold or greater antibody increase to these serotypes, as measured with a protein-A binding assay was observed in 10, 3 and 7 patients, respectively.

Thus, the pneumococcal vaccine was found to induce antibodies against two of the clinically most important, non-vaccine included S. pneumoniae serotypes. The vaccine may not afford substantial increase of opsonic antibodies to serotype 9V pneumococci.

ACKNOWLEDGEMENTS

The studies presented here were made possible by help and encouragement of many persons and were performed at the Department of Infectious Diseases and the Research Department 2, E-blocket, University of Lund, Sweden.

Special thanks are due to:

Håkan Odeberg, my teacher and friend who introduced me to the interaction between phagocytes and microorganisms,

Inge Olsson, who presented me to the field of granulocyte research,
Anders Sjöholm for his careful guidance into the complement system,
Bo Ursing for encouragement and provision of possibilities to start
this investigation,

Folke Nordbring, Karl Emil Thulin and Åke Nordén for their interest
and support,

Rune Grubb and other colleagues at the Department of Medical Micro-
biology for generous support,

Jørgen Henriksen and Freddy Karup Pedersen, Statens Seruminstitut,
Copenhagen for determination of pneumococcal antibodies,

my coauthors and my friends at the Department of Infectious Diseases
and the Research Department 2, who bravely sacrificed their granulo-
cytes for the battle with the pneumococci,

Britt Turesson and Asta Lundquist for pleasant and excellent perfor-
mance of experiments,

Gun Mårtensson for typing and retyping manuscripts.

Financial support was given by the Swedish Medical Research Council,
Medical Faculty, University of Lund, Malmöhus läns landsting and A.
Österlunds stiftelse.

REFERENCES

Agnello, V. *Medicine* 57:1, 1978.

Alestig, K., Hebelka, M., Stenqvist, K. & Svedhem, A. *Läkartidn* 73: 1322, 1976.

Alexander, J.W., Windhurst, D.B. & Good, R.A. *J Lab Clin Med* 72:136, 1968.

Amman, A.J., Addiego, J., Wara, D.W., Lubin, B., Smith, W.B. & Mentzer, W.C. *N Engl J Med* 297:897, 1977.

Amman, A.J., Schiffman, G., Addiego, J.E., Wara, W.M. & Wara, D.W. *Rev Infect Dis* 3:S160, 1981.

Amsbaugh, D.F., Prescott, B. & Baker, P.J. *J Immunol* 121:1483, 1978.

Andersson, B., Eriksson, B., Falsen, E., Fogh, A., Hanson, L.A., Nylén, O., Peterson, H. & Svanborg Edén, C. *Infect Immun* 32:311, 1981.

Appelbaum, P.C., Scragg, J.N., Bowen, A.J., Bhamjee, A., Hallett, A.F. & Cooper, R.C. *Lancet* 2:995, 1977.

Austrian, R. Pneumococcal-type surveillance and the composition of pneumococcal vaccine p. 187-188. In M.T. Parker (ed.), *Pathogenic streptococci*. Reedbooks, Chersey, England, 1979.

Austrian, R. Pneumococci p. 595-606. In B.D. Davis, R. Dulbecco, H.N. Eisen & H. S. Ginsberg (eds.). *Microbiology*. Harper & Row, Publishers Inc., 1980.

Austrian, R. Rev Infect Dis 3:S1, 1981.

Austrian, R., Douglas, R.M., Schiffman, G., Coetzee, A.M., Koornhof, H.J., Hayden-Smith, S. & Reid, R.D. Trans Assoc Am Physicians 89: 184, 1976.

Austrian, R. & Gold, J. Ann Intern Med 60:759, 1964.

Baddiley, J. Proc R Soc B 170:331, 1968.

Broome, C.V., Facklam, R.R., Allen, J.R., Fraser, D.W. & Austrian, R. J Infect Dis 141:119, 1980.

Brown, E.J., Hosea, S.W. & Frank, M.M. J Immunol 126:2230, 1981.

Brown, E.J., Hosea, S.W., Hammer, C.H., Burch, C.G. & Frank, M.M. J Clin Invest 69:85, 1982.

Bulletin of the World Health Organization 39:935, 1968.

Bulletin of the World Health Organization 59:489, 1981.

Callahan, L.T., Woodhour, A.F., Mecker, J.B. & Hilleman, M.R. J Clin Microbiol 10:459, 1979.

Center for Disease Control. Morbidity and Mortality Weekly Report 28:277, 1979.

Corry, J.M., Polhill, R.B., Edmonds, S.R. & Johnston, R.B. Jr. J Pediatr 95:964, 1979.

Davis, C.A. & Forristal, J. J Lab Clin Med 96:633, 1980.

Densen, P. & Mandell, G.L. Rev Infect Dis 2:817, 1980.

Edelson, P.J. Rev Infect Dis 4:124, 1982.

Editorial. Ann Intern Med 90:984, 1979.

Editorial. Lancet II:781, 1980.

Edwards, M.S., Nicholson-Weller, A., Baker, C.J. & Kasper, D.L. J Exp Med 151:1275, 1980.

Ellen, R.P. & Gibbons, R.J. Infect Immun 9:85, 1974.

Ellis, E.F. & Smith, R.T. Pediatrics 37:111, 1966.

Fainstein, V. & Musher, D.M. J Infect Dis 141:172, 1980.

Fearon, D.T. & Austen, K.F. N Engl J Med 303:259, 1980.

Fine, D.P. Infect Immun 12:772, 1975.

Fine, D.P. Complement and Infectious Disease. CRC Press, Inc., Florida, 1981.

Frank, M.M., Hosea, S.W., Brown, E.J. & Hamburger, M.I. N Engl J Med 304:245, 1981.

Gardner, S.E., Anderson, D.S., Webb, B.J., Stitzel, A.E., Edwards, M.S., Spitzer, R.E. & Baker, C.J. Infect Immun 35:800, 1982.

Giebink, G.S., Foker, J.E., Kim, Y. & Schiffman, G. J Infect Dis 141:404, 1980.

Giebink, G.S., Verhoef, J., Peterson, P.K. & Quie, P.G. Infect Immun 18:291, 1977.

Graffner, H., Gullstrand, P. & Hallberg, T. Scand J Haematol 28:369, 1982.

Gray, B.M., Converse, G.M. III & Dillon, H.C. Jr. J Infect Dis 140:979, 1979.

Griffin, F.M. Jr., Griffin, J.A., Leider, J.E. & Silverstein, S.C. J Exp Med 142:1263, 1975.

Guckian, J.C., Christensen, G.D. & Fine, D.P. J Infect Dis 142:175, 1980.

Götze, O., Medicus, R.G. & Müller-Eberhard, H.J. J Immunol 118:525, 1977.

Harboe, M. & Fölling, I. Scand J Immunol 3:471, 1974.

Havas, H.F., Berney, S., Goodis, A. & Schiffman, G. Cancer Res 39:3783, 1979.

Heffron, R. Pneumonia. With special reference to pneumococcus lobar pneumonia. Second printing, Harvard University Press, Cambridge, Massachusetts, 1979.

Hendley, J.O., Sande, M.A., Stewart, P.M. & Gwaltney, J.M. Jr. J Infect Dis 132:55, 1975.

Hilleman, M.R., Carlson, A.J. Jr., McLean, A.A., Vella, P.P., Weibel, R.E. & Woodhour, A.F. Rev Infect Dis 3:531, 1981.

- Holmes, B., Page, A.R. & Good, R.A. J Clin Invest 46:1422, 1967.
- Hosea, S.W., Brown, E.J. & Frank, M.M. J Infect Dis 142:903, 1980.
- Hosea, S.W., Brown, E.J., Hamburger, M.M. and Frank, M.M. N Engl Med 304:245, 1981 a.
- Hosea, S.W., Burch, C.G., Brown, E.J., Berg, R.A. & Frank, M.M. Lancet 1:804, 1981 b.
- Jacob, G.J., Warr, G.A. & Sannes, P.L. Infect Immun 27:960, 1980.
- Johansen, K.S. & Karup Pedersen, F. Acta Path Microbiol Scand. Sect C 90:265, 1982.
- Johnston, R.B. Jr. Rev Infect Dis 3:282, 1981.
- Johnston, R.B. Jr., Klemperer, M.R., Alper, C.A. & Rosen, F.S. J Exp Med 129:1275, 1969.
- Jonsson, M. and Alvin, A. Scand J Infect Dis 3:141, 1971.
- Kaplan, M.H. & Volankis, J.E. J Immunol 112:2135, 1974.
- Karup Pedersen, F. & Henrichsen, J. J Clin Microbiol 15:372, 1982 a.
- Karup Pedersen, F., Henrichsen, J. & Schiffman, G. Acta Paediatr Scand 71:451, 1982 b.
- Klebanoff, S.J. J Exp Med 126:1063, 1967.
- Klein, J.O. Rev Infect Dis 3:246, 1981.

- Kleinerman, E.S., Snyderman, R. & Daniels, C.A. *Lancet* 2:1063, 1975.
- Koch, C. *Acta Path Microbiol Scand. Sect B* 82:136, 1974.
- Krivit, W. *Am J Haematol* 2:193, 1977.
- Kronvall, G. & Williams, R.C. Jr. *J Immunol* 103:828, 1969.
- Käyhty, H. Antibodies to capsular polysaccharides of *Neisseria meningitidis* of groups A and C and *Hemophilus influenzae* type b and their relation to vaccination and disease. Academic dissertation, Helsinki, 1981.
- Lachman, P.J. Complement p. 284-357. In M. Sela (ed.), *The antigens*, Vol V. Academic Press, New York, 1979.
- Larson, H.E. & Blades, R. *Lancet* 1:283, 1976.
- Lee, C.-J., Fraser, B.A., Szu, S. & Lin, K.-T. *Rev Infect Dis* 3:323, 1981.
- Lund, E. & Henrichsen, J. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae* p. 241-262. In T. Bergan & J.R. Norris (eds.), *Methods in microbiology*. Vol. 12. Academic Press, Inc., New York, 1978.
- Maalö, O. On the relation between alexin and opsonin. E. Munksgaard, Copenhagen, 1946.
- Matthews, N., Stark, J.M., Harper, P.S., Doran, J. & Jones, D.M. *Clin Exp Immunol* 39:53, 1980.
- Minor, D.R., Schiffman, G. & McIntosh, L.S. *Ann Intern Med* 90:887, 1979.

- Minta, J.O. & Vetvutanapibul, W. *Immunochemistry* 15:93, 1978.
- Mold, C., Nakayama, S., Holzer, T.J., Gewurtz, H. & DuClos, T.W. *J Exp Med* 154:1703, 1981.
- Mosesson, M.W. & Amrani, D.L. *Blood* 56:145, 1980.
- Mufson, M.A., Kruss, D.M., Wasil, R.E. & Metzger, W.I. *Arch Intern Med* 134:505, 1974.
- Mäkelä, P.H., Sibakov, M., Henrichsen, J., Luotonen, J., Leinonen, M., Timonen, M., Koskela, M., Pukander, J., Grönros, P., Pöntynen, S. & Karma, P. *Lancet* 2:547, 1980.
- Nelson, R.D., Quie, P.G. & Simmons, R.L. *J Immunol* 115:1650, 1975.
- Newman, S.L., Vogler, L.B., Feigin, R.D. & Johnston, R.B. *N Engl J Med* 299:290, 1978.
- Odeberg, H. & Olsson, I. *J Clin Invest* 56:1118, 1975.
- Osler, W.: Lobar pneumonia p. 108-137, In *The principles and practice of Medicine*, 3 ed., Appelton, New York, 1901.
- Pangburn, M.K. & Müller-Eberhard, H.J. *J Exp Med* 152:1102, 1980.
- Petersen, B.H., Lee, T.J., Snyderman, R. & Brooks, G.F. *Ann Intern Med* 90:917, 1979.
- Peterson, P.K. & Quie, P.G. *Ann Rev Med* 32:29, 1981.
- Pitt, J. & Bernheimer, H.P. *Infect Immun* 9:48, 1974.

- Prellner, K. Acta Path Microbiol Scand. Sect C 87:213, 1979.
- Prellner, K. Acta Path Microbiol Scand. Sect C 88:187, 1980.
- Prellner, K. Acta Path Microbiol Scand. Sect C 89:359, 1981.
- Proctor, R.A., Pendergast, E. & Mosher, D.F. Blood 59:681, 1982.
- Radetsky, M.S., Istre, G.R., Johansen, T.L., Parmelee, S.W., Lauer, B.A., Wiesenthal, A.M. & Glode, M.P. Lancet 2:771, 1981.
- Riesen, W.F., Skvaril, F. & Braun, D.G. Scand J Immunol 5:383, 1976.
- Robbins, J.B., Lee, C-J., Rastogi, S.C., Schiffman, G. & Henrichsen, J. Infect Immun 26:1116, 1979.
- Roberts, R.B.: Streptococcus pneumoniae p. 1589. In G.L. Mandell, R.G. Douglas & J.E. Bennett (eds.). Principles and Practice of Infectious Diseases. John Wiley & Sons, Inc., New York, 1979.
- Rosen, F.S. & Janeway, C.A. N Engl J Med 275:709, 1966.
- Root, R.K. & Cohen, M.S. Rev Infect Dis 3:565, 1981.
- Root, R.K., Ellman, L. & Frank, M.M. J Immunol 109:477, 1972.
- Rozing, J., Brons, N.H.C. & Benner, R. Immunology 34:909, 1978.
- Sbarra, A.J. & Karnovsky, M.L. J Biol Chem 234:1355, 1959.
- Scharfstein, J., Correa, E.B., Gallo, G.R. & Nussenzweig, V. J Clin Invest 63:437, 1979.

Schiffman, F. Rev Infect Dis 3:518, 1981.

Schreiner, A., Digranes, A., Myking, O. & Solberg, C.O. Infection 1:137, 1973.

Schulkind, M.L., Ellis, E.F. & Smith, R.T. Ped Res 1:178, 1967.

Schwartz, J.S. Ann Intern Med 96:208, 1982.

Selinger, D.S. & Reed, P.W. Infect Immun 23:545, 1979.

Siber, G.R., Ransil, B.J. & Schiffman, G. Infect Immun 28:641, 1980 a.

Siber, G.R., Schur, P.H., Aisenberg, A.C., Weitzman, S.A. & Schiffman, G. N Engl J Med 303:178, 1980 b.

Siber, G.R., Weitzman, S.A., Aisenberg, A.C., Weinstein, H.J. & Schiffman, G. N Engl J Med 299:442, 1978.

Simberkoff, M.S., Schiffman, G., Katz, L.A., Spicehandler, J.R., Moldover, N.H. & Rahal, J.J. Jr. J Lab Clin Med 96:363, 1980.

Singer, D.B. Perspect Pediatr Pathol 1:285, 1973.

Sloyer, J.L., Ploussard, J.A. & Howie, V.M. Rev Infect Dis 3:5119, 1981.

Smit, P., Oberholzer, D., Hayden-Smith, S., Koornhof, H.J. & Hilleman, M.R. JAMA 238:2613, 1977.

Smith, M.R. & Wood, W.B. J Exp Med 130:1209, 1969.

Snyderman, R. & Goetzl, E.J. Science 213:830, 1981.

Solberg, C.O. *Acta Med Scand* 191:383, 1972.

Stephens, C.G., Williams, R.C. Jr. & Reed, W.P. *Infect Immun* 17:296, 1977.

Stossel, T.P. *N Engl J Med* 290:717, 1974.

Stossel, T.P., Alper, C.A. & Rosen, F.S. *J Exp Med* 137:690, 1973.

Sullivan, J.L., Ochs, H.D., Schiffman, G., Hammerschlag, M.R., Miser, J., Vichinsky, E. & Wedgwood, R.J. *Lancet* 1:178, 1978.

Szu, C.S., Lee, C-J., Parke, J.C. Jr., Schiffman, G., Henrichsen, J., Austrian, R., Rastozzi, S.C. & Robbins, J.B. *Infect Immun* 35:777, 1982.

Tan, J.S., Watanakunakorn, C. & Phair, J.P. *J Lab Clin Med* 78:316, 1971.

Verbruch, H.A., van Dijk, W.C., van Erne, M.E., Peters, R., Peterson, P.K. & Verhoef, J. *Infect Immun* 26:808, 1979.

Volkman, A. *Ser Haematol* 3:62, 1970.

Wara, D.W. *Rev Infect Dis* 3:299, 1981.

Ward, J. *Rev Infect Dis* 3:254, 1981.

White, B. *The biology of pneumococcus*. Second printing, Harvard University Press, Cambridge, Massachusetts, 1979.

Wilkinson, P.C. *Rev Infect Dis* 2:293, 1980.

Winkelstein, J.A. *J Pediatr* 82:747, 1973.

Winkelstein, J.A. Rev Infect Dis 3:289, 1981.

Winkelstein, J.A., Abramovitz, A.S. & Tomasz, A. J Immunol 124:2502, 1980.

Winkelstein, J.A., Bocchini, J.A. Jr. & Schiffman, G. J Immunol 116: 367, 1976.

Winkelstein, J.A., Lambert, G.H. & Swift, A. J Pediatr 87:430, 1975.

Winkelstein, J.A. & Shin, H.S. J Immunol 112:1635, 1974.

Winkelstein, J.A., Shin, H.S. & Wood, W.B. Jr. J Immunol 108:1681, 1972.

Winkelstein, J.A. & Tomasz, A. J Immunol 118:451, 1977.

Winkelstein, J.A. & Tomasz, A. J Immunol 120:174, 1978.

Wood, W.B. Jr., Smith, M.R. & Watson, B. J Exp Med 84:387, 1946.

GRANULOCYTE PHAGOCYTOSIS
AND KILLING OF VIRULENT AND
AVIRULENT SEROTYPES OF
STREPTOCOCCUS PNEUMONIAE

J. H. BRACONIER

and

H. ODEBERG

Lund, Sweden

From the Department of Infectious Diseases,
Department of Internal Medicine, and
Research Laboratory for Clinical
Haematology, University of
Lund, Lund, Sweden

Reprinted from

THE JOURNAL OF LABORATORY AND
CLINICAL MEDICINE
St. Louis

Vol. 100, No. 2, pp. 279-287, August, 1982

(Copyright © 1982 by The C. V. Mosby Company)

(Printed in the U. S. A.)

Granulocyte phagocytosis and killing of virulent and avirulent serotypes of *Streptococcus pneumoniae*

J. H. BRACONIER and H. ODEBERG Lund, Sweden

Five commonly isolated *Streptococcus pneumoniae* serotypes (3, 6, 14, 19, and 23) and five rarely found serotypes (31, 35, 36, 42, and 43) were compared to elucidate whether increased resistance against granulocyte phagocytosis and killing could explain the restricted number of pneumococcal serotypes found in infections. There was a great variation in sensitivity among the serotypes to granulocyte killing. No consistent pattern was found when pathogenicity and resistance to granulocytes were compared. The results do not indicate that the increased tendency of pathogenic pneumococcal serotypes to cause infections is due to increased resistance to granulocytes. Monocyte killing of some pneumococcal serotypes (6, 19, 23, 35, and 43) was also studied and found very similar to granulocyte killing. Defective granulocyte killing of encapsulated pneumococci was due to impaired phagocytosis. Moreover, no correlation was found between the sensitivity of the serotypes to isolated intragranulocytic microbial systems (i.e., MPO, hydrogen peroxide, or CCP) and the sensitivity to killing by intact granulocytes or pathogenicity. The significance of both the classical and the alternative complement pathways for pneumococcal opsonization was indicated by reduced, but residual phagocytosis in C2-deficient and MgEGTA-chelated serum. (J LAB CLIN MED 100:279, 1982.)

Abbreviations: complement factor 2 (C2), chymotrypsin-like cationic proteins (CCP), colony forming units (CFU), enzyme-linked immunosorbent assay (ELISA), Hanks' balanced salt solution (HBSS), N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES), magnesium-ethyleneglycoltetraacetic acid (MgEGTA), myeloperoxidase (MPO)

The pneumococci are the most common bacterial pathogens isolated in otitis media and other respiratory infections. The virulence of *Streptococcus pneumoniae* is considered to be conditioned by the antiphagocytic properties of the capsule.¹ The association between increased virulence and resistance to the phagocytic bactericidal activity of granulocytes has been demonstrated for encapsulated strains of other common pathogens, e.g., *Staphylococcus aureus*² and *Escherichia coli*.³ The pneumococcal capsular polysaccharide displays a great variation, and more than 80 serological types of pneumococci have been differentiated by the immunologically distinct polysaccharides in their capsules.¹ However, only a limited number of serotypes are associated with infectious conditions. In some

From the Department of Infectious Diseases, Department of Internal Medicine, and Research Laboratory for Clinical Haematology, University of Lund, Lund, Sweden.

This investigation was supported by the Swedish Medical Research Council, the Medical Faculty of Lund and by the Foundation of A. Österlund.

Submitted for publication Jan. 5, 1982; accepted Feb. 27, 1982.

Reprint requests: J. H. Bracquier, Department for Infectious Diseases, University Hospital, S-221 85 Lund, Sweden.

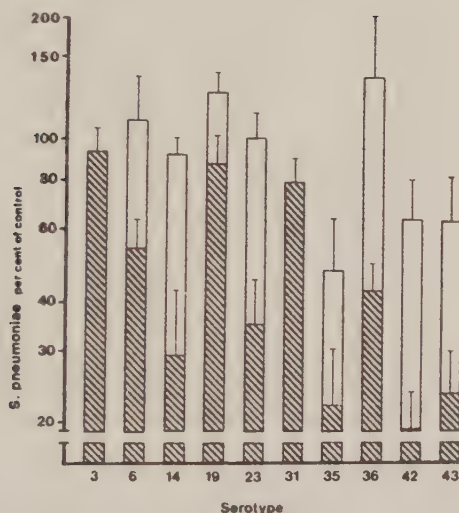


Fig. 1. Survival of pathogenic (3, 6, 14, 19 and 23) and nonpathogenic (31, 35, 36, 42 and 43) serotypes of *S. pneumoniae* after incubation with granulocytes. Granulocytes (10^6) were incubated with about 10^7 bacteria in 0.5 ml of HEPES-HBSS with 4% (open bars) or 8% (cross-hatched bars) pooled serum. Types 3 and 31 only in 8% serum. After 30 min the total number of colony-forming units were determined. Results are shown as means and S.D. of at least six experiments.

recent studies on otitis media, the types 3, 6, 14, 18, 19, and 23 were responsible for about 70% of the pneumococcal infections.⁴⁻⁶ In the present work, granulocyte phagocytosis and killing of five of these serotypes (3, 6, 14, 19, and 23) were compared to killing of five serotypes that rarely cause infections (31, 35, 36, 42, and 43). Monocyte killing of some pneumococcal serotypes was studied. Opsonization of *S. pneumoniae* was also evaluated by the use of MgEGTA-chelated serum, a C2-deficient serum, and an immunoglobulin-deficient serum. The hypothesis of the study was that *S. pneumoniae* serotypes commonly isolated in infections would be more resistant to phagocytosis and killing by granulocytes.

Methods

Informed consent was obtained from the blood donors and the study was approved by the Ethical Committee of the University of Lund.

Bacteria. Clinical isolates of *S. pneumoniae* serotypes 3, 6, 14, 19, and 23 were obtained from the Department of Medical Microbiology, University of Lund, and the types 31, 35, 36, 42, and 43 from Statens Seruminstitut, Copenhagen, Denmark. Bacteria were maintained on blood agar and renewed every fourth to sixth week from stock cultures in fetal calf serum, which were stored at -80°C . On the day of experiment, the bacteria were transferred to a brain-heart infusion broth (Difco Laboratories, Inc., Detroit, Mich.) fortified with 50 $\mu\text{g}/\text{ml}$ cysteine chloride in anaerobic milieu (Gas Pack, Becton, Dickinson & Co., Cockeysville, Md.); the bacteria were harvested in the logarithmic growth phase, washed twice, and suspended in HBSS buffered with 16 mM HEPES, pH 7.4.

Phagocytes. Human granulocytes and mononuclear cells were separated from peripheral blood

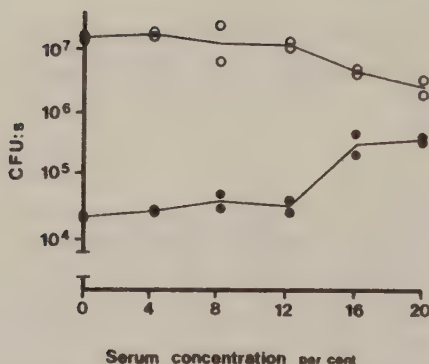


Fig. 2. Survival of *S. pneumoniae* serotype 19 after incubation with granulocytes in different concentrations of pooled serum. About 10^7 bacteria were incubated with 10^6 granulocytes. Total number of viable bacteria (○) were determined after 30 min. Granulocyte associated pneumococci (●) were quantified as detailed in methods. CFU/s, Colony-forming units.

by using Isopaque-Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden). The bottom layer with granulocytes was mixed with 0.87% NH₄Cl for lysis of red cells, washed twice, and suspended in HEPES-HBSS as earlier described.⁶ Mononuclear cells were harvested from the interphase, washed twice, and suspended in HEPES-HBSS. The mononuclear cell suspension contained approximately 15% to 25% monocytes.⁷

Antibacterial granulocyte proteins. CCP⁸ and MPO⁹ were isolated from human granulocytes as earlier described.

Opsonins. Pooled human serum stored at -80°C was used as opsonin source in most of the experiments. Opsonization with defective classical complement pathway was evaluated in serum chelated with 10 mM MgEGTA^{10, 11} and in serum from a patient with deficiency of C2. In some experiments serum from a patient with common variable hypogammaglobulinemia was used. Specific pneumococcal antibodies in this serum were kindly determined by Dr. J. Henriksen, Statens Seruminstitut, Copenhagen with ELISA. In spite of very low immunoglobulin levels (IgG 0.9 gm/L, IgA 0.09 gm/L, and IgM 0.09 gm/L), the titers of antibodies to serotypes 3, 6A, 14, 19F, and 23F were about the same as in normal sera.

Ingestion and killing of pneumococci by phagocytes. About 1×10^7 bacteria were incubated with desired concentrations of serum in HEPES HBSS for 5 min at 37°C before addition of 10^6 granulocytes or 10^7 mononuclear cells. The total volume was 0.5 ml. Incubations were at 37°C in plastic capped tubes, during rotation end-over-end at a rate of 20 rpm (granulocytes) or in a shaking water bath (monocytes). Controls were run without phagocytes. Aliquots of 20 μl were removed at 0 and 30 min and diluted in 8 ml of distilled water containing 0.05% bovine serum albumin. Viable bacteria were determined by colony counting with the pour-plate method.

For determination of granulocyte-associated colony-forming units, 2.5 ml of cold ($+4^{\circ}\text{C}$) HEPES-HBSS were added. The granulocytes were washed twice by centrifugation at $160 \times g$ for 5 min at $+4^{\circ}\text{C}$ ¹² and the number of colony-forming units in the cell pellet was determined.

Bactericidal activity of CCP and of a MPO-hydrogen peroxide system. About 10^6 *S. pneumoniae* per milliliter were incubated at 37°C for 30 min in a modified HEPES-HBSS buffer with 58 $\mu\text{g/ml}$ CCP or 10 $\mu\text{g/ml}$ MPO and 0.8 $\mu\text{g/ml}$ glucose oxidase (Hughes & Hughes Ltd, Romford Essex, England) for continuous generation of H₂O₂.^{13, 14} Viable bacteria were quantified as described above.

Data analysis. Results are expressed as surviving bacteria (percent of controls) in incubations with granulocytes. The number of experiments, mean, S.D. and/or range are given in each figure.

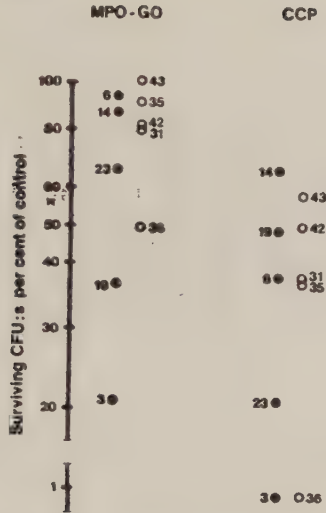


Fig. 3. Bactericidal activity of granulocyte constituents on pathogenic (●) and nonpathogenic serotypes (○) of *S. pneumoniae*. About 10^6 pneumococci were incubated in a modified HEPES-HBSS with 58 $\mu\text{g}/\text{ml}$ CCP or 10 $\mu\text{g}/\text{ml}$ MPO and 0.8 $\mu\text{g}/\text{ml}$ glucose oxidase. Incubations were at 37° C for 30 min before determination of surviving bacteria. Results are given as means of two experiments CFU's, Colony-forming units; GO, glucose oxidase.

Results

Granulocyte phagocytosis and killing of *S. pneumoniae*. Fig. 1 shows granulocyte killing of five *S. pneumoniae* serotypes (3, 6, 14, 19, and 23) commonly encountered in infections and of five rarely isolated serotypes (31, 35, 36, 42, and 43) in 4% or 8% pooled serum. There was a great variation in sensitivity among the serotypes to granulocytes. With 4% serum only three serotypes, all nonpathogenic (serotypes 35, 42, and 43), displayed a substantial reduction of colony-forming units after 30 min incubation together with granulocytes. Granulocyte killing was considerably more efficient in 8% serum, with elimination of about three fourths of *S. pneumoniae* serotypes 35, 42, and 43. Also the pathogenic serotypes 6, 14, and 23 were rather efficiently killed by granulocytes at this concentration of serum. However, very little reduction of two pathogenic serotypes (3 and 19) and of one nonpathogenic serotype (31) was found. By an increase in the concentration of serum, more efficient granulocyte killing could be obtained. Thus serotype 19 was readily killed when the concentration was raised to 16% and 20% (Fig. 2). No reduction of serotype 3 was, however, found even in the presence of 80% serum (results not shown).

The inefficient granulocyte killing of pneumococci at low serum concentrations was due to defective phagocytosis, since the granulocyte-associated microorganisms accounted for a minority of the surviving bacteria. The granulocyte-associated bacteria accounted for

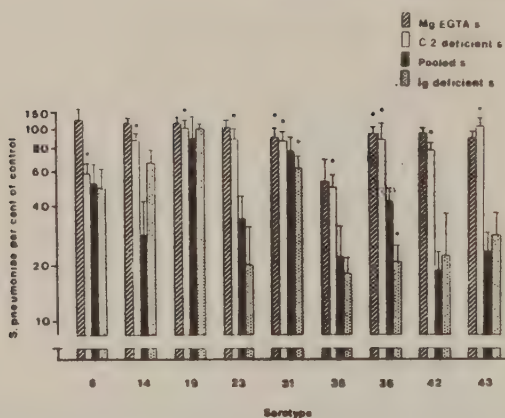


Fig. 4 Opsonization of *S. pneumoniae* in MgEGTA-chelated pooled serum, C2-deficient serum, pooled serum, and immunoglobulin-deficient serum. Granulocytes (10^6) were incubated with about 10^7 pneumococci in 0.5 ml of HEPES-HBSS with 8% serum. Surviving bacteria were quantified after 30 min. Results are shown as means and S.D. of four to six experiments. Asterisk, bars and brackets represent means and ranges of only two experiments; s, serum.

$1.6\% \pm 1.6$ (mean $\pm$ S.D. of 28 experiments, including all serotypes except 3 and 31) of the total number of surviving organisms after incubations in 4% serum. In 8% serum the corresponding figure for 30 experiments, including all serotypes, was $5.4\% \pm 4.9$. Thus the fraction of granulocyte-associated bacteria increased somewhat, with more efficient phagocytosis at the higher serum concentration. The experiment with serotype 19 depicted in Fig. 2 illustrates the effect of increased serum concentration on both granulocyte killing and on the fraction of granulocyte-associated pneumococci.

Effect of isolated granulocyte antibacterial agents on the viability of *S. pneumoniae*. There was a great variation among the serotypes of *S. pneumoniae* in susceptibility to both the MPO-hydrogen peroxide system and the CCP (Fig. 3). No correlation to pathogenicity or susceptibility to intact granulocytes was found. The results support the concept of pneumococcal granulocyte resistance being primarily dependent on defective phagocytosis.

Granulocyte phagocytosis and killing of *S. pneumoniae* in MgEGTA-chelated serum, a C2-deficient serum, and an immunoglobulin-deficient serum. Granulocyte killing of most *S. pneumoniae* serotypes was reduced in serum with defective classical complement pathway due to MgEGTA chelation (Fig. 4). In higher serum concentrations substantial bacterial killing was obtained in the chelated serum with both pathogenic and nonpathogenic serotypes as illustrated in Fig. 5 with serotypes 6, 14, 23, and 35.

Compromised classical complement pathway due to C2 deficiency also resulted in defective granulocyte killing of *S. pneumoniae* (Fig. 4). In serum from a patient with common variable hypogammaglobulinemia, the phagocytosis of pneumococci was, however, as efficient as in pooled serum.

Monocyte killing of *S. pneumoniae*. Monocyte killing of serotypes 6, 19, 23, 35, and

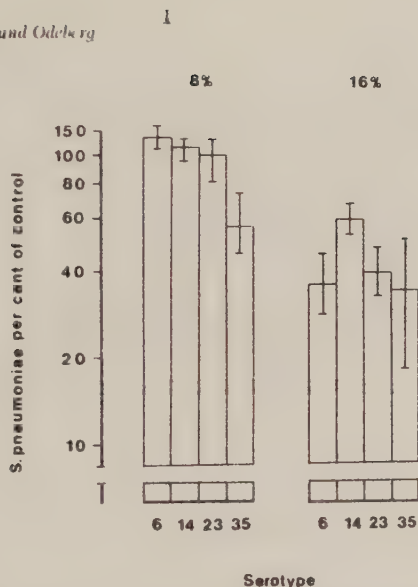


Fig. 5 Opsonization of *S. pneumoniae* (serotypes 6, 14, 23, and 35) in different concentrations of MgEGTA-chelated serum. Granulocytes (10^6) were incubated with about 10^7 pneumococci in 8% or 16% serum chelated with 10 mM MgEGTA. Viable bacteria were determined after 30 min. Results are shown as means and ranges of two to six experiments.

43 is shown in Fig. 6. The susceptibility of the *S. pneumoniae* serotypes to killing by mononuclear cells was found very similar to killing by granulocytes. Thus serotype 35 was most sensitive to monocytes and serotype 19 was not killed even at 16% serum.

Discussion

The present work has shown a considerable heterogeneity among encapsulated *S. pneumoniae* in their resistance to granulocyte killing. The studies could not confirm our hypothesis that the tendency to cause infections might be determined by the degree of resistance to phagocytosis and killing by granulocytes, since some serotypes that rarely cause infections (serotypes 31 and 36) were as resistant as some commonly recovered serotypes. Thus other interpretations of the variable tendency among *S. pneumoniae* serotypes to cause infections must be of considerable importance.

Microbial killing in granulocytes includes the participation of reactive compounds formed at phagocytosis-induced oxidative metabolism as well as microbicidal constituents of the cytoplasmic granules.¹² Granulocyte MPO constitutes, together with hydrogen peroxide and a halide, a potent intragranulocytic microbicidal system.¹³ The significance of hydrogen peroxide for granulocyte killing of *S. pneumoniae* has been demonstrated by reduced killing by hydrogen peroxide-deficient mutants in granulocytes from patients with chronic granulomatous disease.¹⁴ We have described a bactericidal activity, in particular on gram-positive organisms, of a group of CCPs of human granulocytes.¹⁵ In the

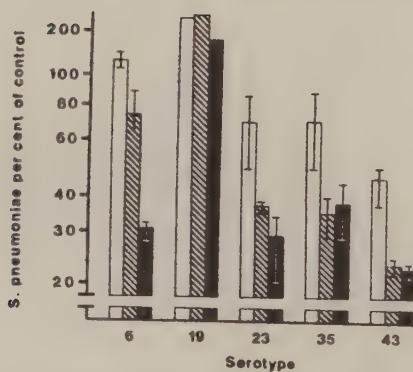


Fig. 6. Survival of *S. pneumoniae* (serotypes 6, 19, 23, 35 and 43) after incubation of about 10^7 bacteria with 1.1×10^7 mononuclear cells in 4% (open bars), 8% (cross-hatched bars), and 16% (solid bars) pooled serum. Viable bacteria were determined after 30 min. Results are shown as means and ranges of two to four experiments (serotype 19 only one experiment).

present study we found a great variability among encapsulated *S. pneumoniae* serotypes in sensitivity to both MPO-hydrogen peroxide and these cationic proteins. There was, however, no correlation between sensitivity of serotypes to these antibacterial systems and sensitivity to killing by intact granulocytes or pathogenicity. The results indicate that variations in pathogenicity or resistance to granulocytes of different serotypes of *S. pneumoniae* do not depend on variable sensitivity to these intragranulocytic bactericidal systems. However, the results with isolated intragranulocytic microbicidal systems should be interpreted with caution because the exact mechanisms of microbial killing in granulocytes have not been clarified.¹⁵

The defective granulocyte killing of *S. pneumoniae* observed in our study was due to impaired phagocytosis. Thus, in experiments with absent or low pneumococcal killing, only a very small number of surviving *S. pneumoniae* were found to be cell-associated after differential centrifugation and washing of the granulocytes. When bacterial killing was more efficient, the number of cell-associated bacteria increased but still constituted only a minority of surviving organisms.

By an increase in the concentration of serum, all serotypes of *S. pneumoniae* tested, except serotype 3, were efficiently phagocytized and killed by granulocytes. These results suggest that differences among serotypes of encapsulated *S. pneumoniae* in resistance to the phagocytic bactericidal activity of granulocytes are due to differences in the interaction with or in the requirements of serum opsonins, i.e., immunoglobulins and activated complement.¹⁷

Our study showed reduced opsonic activity for the pathogenic and nonpathogenic pneumococcal serotypes in MgEGTA-chelated and C2-deficient sera. The results demonstrate the participation of the classical complement pathway opsonization in granulocyte killing of *S. pneumoniae*. The results do not indicate classical pathway activation to be decisive for the varying pathogenicity of pneumococcal serotypes. Giebink et al.¹⁸ have earlier found reduced granulocyte phagocytosis of several pneumococcal serotypes (6, 18,

23, and 25) in C2-deficient and MgEGTA-chelated serum. In addition they noticed reduced *S. pneumoniae* opsonization in immunoglobulin-deficient serum, which is in accordance with the concept of the initiation of the classical complement pathway by immunoglobulins.¹⁹ We found, however, that serum from a patient with pronounced hypogammaglobulinemia supported *S. pneumoniae* phagocytosis as efficiently as did pooled serum. This might imply an immunoglobulin-independent classical pathway opsonization of *S. pneumoniae*. In fact, activation of the classical pathway independent of immunoglobulins has been described for pneumococci in the presence of C-reactive protein²⁰ and calcium, which would be blocked by MgEGTA chelation. In our immunoglobulin-deficient serum, however, it is possible that the amounts of specific pneumococcal antibodies were sufficient for immunoglobulin-dependent classical complement activation. The level of pneumococcal antibodies in the immunoglobulin-deficient serum used by Giebink et al.¹⁹ was not stated.

Activation of the alternative pathway by pneumococci has been repeatedly demonstrated^{10, 21, 22} and could explain our findings of opsonization in higher concentrations of chelated serum. At lower concentrations of serum the alternative pathway might have been inhibited by dilution.¹⁹ Fine¹⁰ has suggested that the varying pathogenicity of pneumococcal serotypes is associated with dissimilarities in ability to activate the alternative pathway. However, in an extensive study, Stephens et al.²¹ demonstrated a great variability in alternative pathway activation by both pathogenic and nonpathogenic pneumococci.

Splenectomized individuals convey an increased danger of overwhelming pneumococcal infections,²³ indicating the participation of the monocyte-macrophage system in the defence against *S. pneumoniae*. In the present study we found serum demands for monocyte killing of *S. pneumoniae* to be rather similar to the requirements for granulocyte killing. The results do not indicate that differences in pathogenicity are due to variable resistance to monocytes. However, different elements of the monocyte-macrophage system might have varying requirements for opsonins. Thus increased amounts of antibodies were needed for intravascular pneumococcal clearance in splenectomized animals.²⁴ Also serotypes other than those tested by us may show specific virulence in asplenic individuals, as has been shown in a small study.²⁵

Epidemiological studies in infants have demonstrated that the serotypes causing pneumococcal diseases were similar to commonly carried types.^{26, 27} It is possible that surface factors, possibly associated with capsular structures, facilitate or interfere with the colonization of the mucosa and are decisive for the pathogenicity of pneumococcal serotypes. Similar mechanisms have been suggested for other pathogens.²⁸

The technical assistance of Mrs. Asta Lundquist and Mrs. Britt Turesson is greatly appreciated.

REFERENCES

1. McCarty M. Pneumococci. In: Microbiology, Davis BD, Dulbecco R, Eisen HN, Ginsberg HS, Wood WB, and McCarty M, editors. New York, 1973. Harper & Row, Publishers, p. 694.
2. Melly MA, Duke LJ, Liao D-F, and Hashi JH. Biological properties of the encapsulated *Staphylococcus aureus* M. Infect Immun 10:389, 1974.
3. Rotimi G, Ori P, Soranzo MR, and Patriarca P. Correlation between phagocytic activity and metabolic response of polymorphonuclear leukocytes towards different strains of *Escherichia coli*. Infect Immun 11:417, 1975.
4. Gray BM, Converse GM III, and Dillon HC Jr. Pneumococcal serotypes in childhood infections. In: Pathogenic Streptococci, Parker MT, editor. Chertsey, Surrey, England, 1979. Reedbooks, Ltd., p. 189.
5. Skoyer JL, Howie VM, Ploussard JH, Amman AJ, Austrian R, and Johnston RB Jr. Immune

- response to acute otitis media in children. I. Serotypes isolated and serum and middle ear fluid antibody in pneumococcal otitis media. *Infect Immun* 9:1028, 1974
6. Odeberg H, Olofsson T, and Olsson I: Granulocyte function in chronic granulocytic leukemia I. Bactericidal and metabolic capabilities during phagocytosis in isolated granulocytes. *Br J Haematol* 29:427, 1975
7. Svensson B: Methodological studies of monocyte yeast cell phagocytosis. *Scand J Rheumatol Suppl* 31:5, 1980.
8. Olsson I and Venge P: Cationic proteins of human leukocytes. II. Separation of the cationic proteins of the granules of leukemic myeloid cells. *Blood* 44:235, 1974
9. Olsson I, Olofsson T, and Odeberg H: Myeloperoxidase-mediated iodination in granulocytes. *Scand J Haematol* 9:483, 1972
10. Fine DP: Pneumococcal type-associated variability in alternate complement pathway activation. *Infect Immun* 12:772, 1975.
11. Forsgren A and Quie PG: Influence of the alternative complement pathway on opsonization of several bacterial species. *Infect Immun* 10:402, 1974
12. Verhoef J, Peterson PK, and Quie PG: Kinetics of staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes: a quantitative assay using ³H-thymidine labeled bacteria. *J Immunol Methods* 14:303, 1977.
13. Odeberg H and Olsson I: Antibacterial activity of cationic proteins from human granulocytes. *J Clin Invest* 56:1118, 1975.
14. Odeberg H and Olsson I: Microbicidal mechanisms of human granulocytes: synergistic effects of granulocyte elastase and myeloperoxidase or chymotrypsinlike cationic protein. *Infect Immun* 14:1276, 1976.
15. Klebanoff SJ: Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. *Semin Hematol* 12:117, 1975.
16. Pitt J and Bernheimer HP: Role of peroxide in phagocytic killing of pneumococci. *Infect Immun* 9:48, 1974.
17. Stossel TP: Phagocytosis. *N Engl J Med* 290:717, 1974.
18. Giebink GS, Verhoef J, Peterson PK, and Quie PG: Opsonic requirements for phagocytosis of *S. pneumoniae* type 6, 18, 23 and 25. *Infect Immun* 18:291, 1977.
19. Lachmann PJ: Complement. In: *The Antigens*, Sela M, editor. New York, 1979, Academic Press, Inc., p. 283.
20. Kaplan MH and Volanakis JE: Interaction of C-reactive protein complexes with the complement system. *J Immunol* 112:2135, 1974.
21. Stephens CG, Wilhams RC Jr, and Reed WP: Classical and alternative complement pathway activation by pneumococci. *Infect Immun* 17:296, 1977
22. Prellner K: Complement activation by pneumococci associated with acute otitis media. *Acta Pathol Microbiol Scand [C]* 87:213, 1979
23. Singer DB: Postsplenectomy sepsis. In: *Perspectives in Pediatric Pathology*, Rosenberg HS and Bolander RP, editors. Chicago, 1973, Yearbook Medical Publishers, Inc., p. 285
24. Hosea SW, Brown EJ, Hamburger MI, and Frank MM: Opsonic requirements for intravascular clearance after splenectomy. *N Engl J Med* 304:245, 1981.
25. Gopal V and Bisno AL: Fulminant pneumococcal infections in "normal" asplenic hosts. *Arch Intern Med* 137:156, 1977.
26. Nemir RL, Andrews ET, and Vinograd J: Pneumonia in infants and children. A bacteriologic study with special reference to clinical significance. *Am J Dis Child* 51:1277, 1936
27. Gray BM, Converse GM III, and Dillon HC Jr: Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage and infection during the first 24 months of life. *J Infect Dis* 142:923, 1980
28. Peterson PK and Quie PG: Bacterial surface components and the pathogenesis of infectious diseases. *Annu Rev Med* 32:29, 1981.

Properdin deficiency in a family with fulminant meningococcal infections

A. G. SJÖHOLM, J.-H. BRACONIER & C. SÖDERSTRÖM *Department of Infectious Diseases, University Hospital, Lund; Department of Immunology, Institute of Medical Microbiology and Research Laboratory for Clinical Haematology, University of Lund, Sweden*

(Accepted for publication 4 June 1982)

SUMMARY

Three males in a large family showed a selective deficiency of properdin (P). One of the P deficient individuals died from a fulminant infection with *Neisseria meningitidis* group C. The family history revealed three previous cases of similar infections with a fatal outcome. The deficiency did not appear to be associated with repeated bacterial infections. The pattern of inheritance suggested an X-linked mode of transmittance. However, heterozygous carriers were not clearly distinguished in the family. P deficient serum supported immune haemolysis in a normal fashion. Alternative pathway functions, such as the activation of C3 by inulin or zymosan, lysis of guinea-pig erythrocytes in agarose gel and opsonization of endotoxin coated oil particles, were grossly impaired in P deficient serum while efficient C3 activation was produced by addition of cobra venom factor.

INTRODUCTION

Deficiencies inherited as autosomal recessive traits have been recognized for several of the proteins belonging to the complement system. Defects of the classical pathway components C1, C4 or C2 appear to predispose to development of immune complex diseases such as SLE, and recurrent infections with pyogenic bacteria, including meningococci, are encountered in C3 deficiency syndromes (Alper *et al.*, 1972; Thompson & Lachmann, 1977; Agnello, 1978). Deficiencies of the terminal components C5, C6, C7 or C8 are associated with recurrent bacteremia caused by *Neisseria meningitidis* or *N. gonorrhoeae* (Peterson *et al.*, 1979; Snyderman *et al.*, 1979).

The alternative pathway of complement activation is known as a mechanism for amplification of C3 activation through factor B, factor D and properdin (P) controlled by the factors I and H, and is considered to provide non-immune defence against microbial infection (Fearon & Austen, 1980). In the present study we describe a large family in which three maternally related males were P deficient. One of these died from a fulminant infection with *N. meningitidis* group C. Previously, fatal infections had occurred at different times in three male members of the family. The P deficiency was documented and studied in relationship to alternative pathway functions.

CASE REPORTS

Case 1 (III-10) The index patient was a previously healthy 15 year old boy who was admitted to hospital with high fever and in shock 6 hr from the onset of disease. In the hospital he developed

Correspondence: Anders G. Sjöholm, MD, Department of Immunology, Institute of Medical Microbiology, Sölvegatan 23, S-223 62 Lund, Sweden.

petechial bleedings and was treated with antibiotics and hydrocortisone. The patient died after 5 hr of hospitalization. Cultures from spinal fluid and blood showed *N. meningitidis* type C.

Case 2 (III:8). The previously healthy 6 year old brother of III:10 died in hospital before treatment was given, within 24 hr from the onset of headache, high fever and petechial bleedings. *N. meningitidis* was cultured from the blood. Necropsy revealed purulent meningitis, haemorrhagic pneumonia and bilateral haemorrhagic necrosis of the adrenals.

Case 3 (II:5). This patient was previously healthy with the exception of a pneumonia at the age of 33. At 36 years of age he fell ill with fever, confusion and a petechial rash and died after 28 hr, in spite of treatment with antibiotics and hydrocortisone. Necropsy showed purulent meningitis and generalized purpuric lesions.

Case 4 (II:1). In 1934, this previously healthy 6 year old boy fell ill with nausea, fever and headache. The next day he was unconscious and died before arrival at hospital. The cause of death was considered to be septicaemia.

Case 5 (III:6) This 25 year old male has been healthy with exception for two episodes of meningitis. Cultures were negative and the spinal fluid glucose normal in both instances. The clinical and laboratory examination carried out at the time of the investigation gave no indication of disease.

Case 6 (II:15) This individual is a 40 year old healthy male. Recurrent infections or other disease manifestations were not present in the case history. The physical examination, and laboratory tests were normal.

Further family data. The family history revealed no further cases with severe or recurrent infections, or with other diseases. I:1, I:3 and I:5 reached high age and died from apparently unrelated causes.

MATERIALS AND METHODS

Serum and plasma. Serum and EDTA plasma samples were stored in aliquots at -80°C . A single sample obtained during meningococcal septicaemia was available from the patient III:10. Three samples were drawn from III:6 and one from II:15.

Plasma protein levels. C1q, C1r, C1s, C2, C3, C4, C5 factor B, P, and C1 inactivator (C1 IA) levels were determined by electroimmunoassay (Sjöholm, 1975; Laurell, Mårtensson & Sjöholm, 1978). The same technique was used for measurement of C6, C7, C8, factor I, factor H, the C4 binding protein and C-reactive protein. C9 was demonstrated by double diffusion using commercial anti-C9 (Behringwerke A.G., Frankfurt am Main, W. Germany). Factor D was determined by haemolysis in gel (Martin *et al.*, 1976). IgG, IgA and IgM were quantified by electroimmunoassay (Grubb, 1970, 1974) and IgE by PRIST (Pharmacia Diagnostics, AB, Uppsala, Sweden).

Complement functions and activation. Screening for complement deficiencies was performed by haemolysis in gel using sensitized sheep erythrocytes for the classical, and guinea-pig erythrocytes in magnesium ethylene glycol tetraacetic acid (MgEGTA) for the alternative pathway (Truedsson, Sjöholm & Laurell, 1981). Immune haemolysis was also examined by measurement of the CH₅₀ (Rapp & Borsos, 1970). Granulocyte uptake of Oil Red O coloured di-isodecylphthalate emulsified in endotoxin was measured as described by Stossel, Alper & Rosen (1973) with some modifications (Olofsson, Odeberg & Olsson, 1976). Inulin (BDH Chemicals Ltd., England), highly purified cobra venom factor (CVF) (kindly provided by Dr G. Eggertsen, Uppsala, Sweden) and zymosan (Koch-Light laboratories Ltd, England) were used as activators of the alternative pathway. The zymosan was handled as described by Götze, Medicus & Müller-Eberhard (1977). The cleavage of C3 in the sera was studied by crossed immunoelectrophoresis (Ganrot, 1972). In some experiments the results were evaluated by planimetry, giving the proportion of cleaved C3, mainly C3c, as a percentage of the area covered by precipitate. Crossed immunoelectrophoresis of C1 subcomponent complexes (Laurell, Mårtensson & Sjöholm, 1977) was used for estimation of C1r-C1s-C1-IA complexes in serum.

Purified P. P, in native form, was prepared as described by Götze *et al.* (1977), with omission of the final immune absorption step. The preparation was gel filtrated on Sepharose CL-6B and on Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden). The purified P contained small

amounts of IgG as the only known contaminant. The concentration of P was estimated immunochemically assuming that the pooled reference serum contained P at 27.5 mg/l (Davis & Forristal, 1980).

RESULTS

Complement components

In three individuals (II:15, III:6 and III:10) P was undetectable in serum and plasma by electroimmunoassay. With undiluted samples the finding implied values that were below 2% of the normal, or less than 0.55 mg/l assuming a normal mean for P of 27.5 mg/l (Davis & Forristal, 1980). III:10 showed concentrations of C1q, C4 and C5 in the low normal range and a moderately reduced C2. By crossed immunoelectrophoresis, the serum was found to contain C1r-C1s-C1-1A complexes in clearly increased amounts. The concentrations of other complement components tested were normal or raised in the P deficient individuals (Table 1).

The levels of P, C1q, C1s, C3 and C4 were determined in the four generations of the family studied. The pedigree, including the P values is given in Fig. 1. With the reference area used, clearly decreased P was found in three females (II:3, II:19 and III:17), and in the 5 month old son (IV:1) of III:6. The low values for P, and also for C1q, in this child were considered normal for his age (Davis, Vallota & Forristal, 1979). The P levels were normal in the parents of III:10 (II:11 and II:12) and in those of III:6 (II:9 and II:10), as well as in the father of II:15 (I:4). The values obtained for C1q (50-140%), C1s (94-154%), C3 (81-155%) and for C4 (62-200%) were largely normal in the family members.

Table 1. Complement component levels in percentage of normal and the CH50 in the sera of three properdin deficient individuals. Complement components were measured by electroimmunoassay unless otherwise indicated

	III:10	III:6	II:15	Reference values*
C1q	74	129	183	78-130
C1r	—†	166	149	71-133
C1s	88	114	167	79-139
C4	56	113	83	53-207
C2	50	101	118	75-163
C3	91	128	180	70-136
C5	69	105	98	72-171
C6	110	97	100	55-148
C7	90	119	110	56-146
C8	58	92	50	30-175
C9‡	yes	yes	yes	yes
factor B	108	141	143	59-154
factor D§	160	100	145	36-160¶
properdin	<2	<2	<2	57-153
C1 inactivator	82	136	150	72-153
factor I	105	105	140	54-142
factor H	80	110	142	61-150
C4 binding protein	98	97	141	58-102¶
CH50 (U/ml)	not done	50	30	30-40¶

* The 95% confidence interval as determined in 100 adults.

† Diffuse precipitate.

‡ Double diffusion.

§ Haemolysis in gel assay.

¶ Observed range in 25 adults.

Complement functions

In screening tests for the classical and alternative pathways by haemolysis in gel, all the sera from adult family members, except the sera of II:15, III:6 and III:10, produced normal patterns of lysis. Sera lacking P gave partial lysis in the assay for the alternative pathway, but full lysis in the classical pathway test. With addition of purified P, the P deficient sera gave full lysis in both assays. P deficient serum gave high or normal values in the CH₅₀ test (Table 1). Incubation at 37 °C of sera from the healthy P deficient (II:15 & III:6) in the presence of MgEGTA and zymosan did not result in efficient C3 cleavage such as seen in the control sera. Also, the background C3 cleavage was markedly low in the P deficient sera. Addition of purified P at a physiological concentration normalized the background C3 conversion and the capacity to support C3 activation by zymosan. One experiment is shown in Fig. 2.

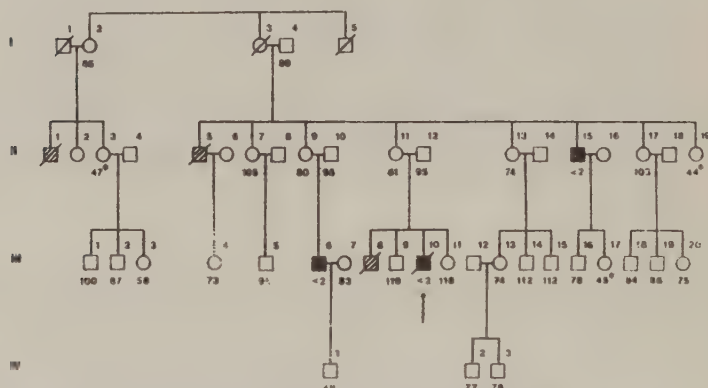


Fig. 1. Pedigree of family with properdin deficiency. Generations are indicated by Roman numerals, individuals in each generation by numbers. Males are represented by squares and females by circles. Deceased members of the family are indicated by an oblique line across the symbol. Closed symbols denote individuals with a complete deficiency of properdin, the index patient is marked with an arrow. Hatched symbols are used for individuals previously dead in fulminant infections. The properdin values in percentage of normal are given below the symbols. Values lower than 2 s.d. of the normal mean (adults) are marked with asterisks.

Like zymosan, inulin did not produce efficient C3 activation in P deficient serum unless P was added. In contrast, incubation with CVF gave C3 cleavage to the same extent in P deficient sera as in the control sera. Unchelated sera were used in these experiments. The sera from II:15 and III:6 showed low background C3 conversion, which was corrected by addition of purified P. Serum from the patient with meningococcaemia III:10 contained cleaved C3 and showed more C3 conversion than the other sera on the addition of P, possibly indicating the presence of complement activating substances in the serum (Table 2). P deficient serum failed to opsonize endotoxin coated oil particles for granulocyte phagocytosis in a normal fashion (Table 3). Addition of P at 23 mg/l and also at 3 mg/l largely restored the uptake to that obtained in the presence of normal sera. Addition of P to the pooled normal serum used as reference did not influence opsonization.

Other plasma proteins

With exception for a high IgE value in III:6 the immunoglobulin levels were normal in the three P deficient individuals with IgG at 6.9–12.7 g/l, IgA at 0.5–1.6 g/l and IgM at 0.5–1.5 g/l and in several of the family members investigated. C-reactive protein was slightly raised in III:10.

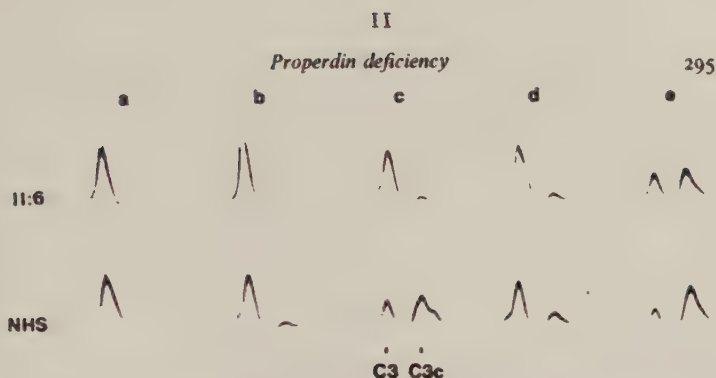


Fig. 2. Analysis by crossed immunoelectrophoresis of the C3 cleavage in properdin deficient serum (II:6) and in normal human serum (NHS) after incubation with zymosan at 37°C for 1 hr. Pelleted zymosan was suspended in the sera at 2 mg/ml. The incubation was carried out in the presence of Mg^{++} (2 mmol/l) and EGTA (10 mmol/l). (a) serum + buffer, 0°C; (b) serum + buffer, 37°C; (c) serum + buffer + zymosan, 37°C; (d) serum + properdin, 37°C and (e) serum + properdin + zymosan, 37°C. The properdin, in native form, was added at a final concentration of 29 mg/l with respect to serum. The final concentration of serum in the incubation mixtures was 75%.

Table 2. Cleavage of C3 in properdin deficient sera incubated with inulin or with cobra venom factor (CVF) at 37°C for 30 min. The values were given in percentage C3 conversion as assessed by crossed immunoelectrophoresis

Volumes in reaction mixtures				
20 μ l	2 μ l	5 μ l	III:10	III:6 II:15 Controls
Serum + NaCl + Tris			26	5 6 19-37§
Serum + inulin* + Tris			28	14 34 51-79¶
Serum + NaCl + properdin†			54	22 16 33-38§
Serum + inulin + properdin			74	60 77 77-89§
Serum + CVF‡ + Tris			not done	66 81 63-86§
Serum + CVF + properdin			not done	76 90 not done

* Inulin at 100 mg/ml suspended in 0.15 M NaCl.

† Purified properdin (160 mg/l) in 0.1 M Tris, 0.4 M NaCl, pH 7.4.

‡ Purified CVF (160 mg/l).

§ Range in five healthy controls.

¶ Range in 18 healthy controls.

DISCUSSION

In II:15 and III:6 the P deficiency could not be attributed to disease. We found no other immunological defect. Alternative pathway dysfunctions in the sera could be corrected with purified P. CVF gave efficient C3 cleavage in the sera, suggesting that additional defects of the alternative pathway were not present.

The P deficient patient III:10 showed evidence of classical pathway activation in the course of a fatal meningococcal infection. The P deficiency might have been present also in II:1, II:5 and III:8, who died of fulminant infections.

Table 3. Uptake of endotoxin coated oil by control granulocytes in the presence of properdin deficient sera

Opsonin source	Uptake of oil*
Control sera†	Range 75-135
III:6 serum	11
III:6 serum + properdin‡	61
II:10 serum	5
II:10 serum + properdin‡	43

* Uptake is given as a percentage of the uptake in pooled control serum.

† $n=7$.

‡ Purified properdin added to serum at a physiological concentration (23 mg/l).

On the assumption that the deficiency was inherited, the most likely mode of transmission was X-linked. Only males were affected, and to our knowledge, the fathers of II:15, III:6 and III:10 were unrelated. The half normal P levels in three females on the maternal side (II:3, II:19 and III:17) and perhaps also in III:3 would fit this model. The half normal value in [V:1], a 5 month old boy, could be explained by his low age (Davis *et al.*, 1979). However, the normal P levels in the parents of the deficient males made the genetic analysis uncertain. In families with a partial P deficiency, the defect appeared to be inherited as an autosomal recessive trait (Davis & Forristal, 1980; Wyatt, Julian & Galla, 1981).

According to current concepts, the only action of P is to stabilize the amplification C3 convertase C3bBb (Fearon & Austen, 1980). Thus, in P deficiency a number of biological functions dependent on efficient C3 activation may be impaired. The observation that incubation at 37°C gave less C3 cleavage in P deficient than in normal sera may imply a role of P in the fluid phase initiation of the alternative pathway.

The fairly well established disease associations of complement deficiency states have suggested a role of the alternative pathway in resistance to bacterial infections (Fearon & Austen, 1980). The present observations indicate that P deficiency may have predisposed to the fulminant infections in the family studied. None of the family members gave evidence of a general susceptibility to bacterial disease with recurrent infections.

The fulminant infections were caused by meningococci in at least two of the patients (III:8 & III:10). It is not known if protective antibodies (Goldschneider, Gotschlich & Artenstein, 1969; Robbins, 1978) were present in the patients, and the definite role of P in this situation has yet to be decided. Abnormal immune responses in members of the P deficient family cannot be excluded and would complicate the interpretation of the association between P deficiency and the infections seen.

Deficiencies of P are probably not common. On the other hand, studies of P levels in health and disease have hardly been extensive. In screening for complement deficiencies, defects of P will be overlooked, unless suitable tests for alternative pathway function are employed.

Supported by grants from the Swedish Medical Research Council (B79-16X-05430-01 and B81-03X-00581-17A), the Medical Faculty, University of Lund, Forssman's Foundation, Österlund's Foundation, T. & E. Segerfalk's Foundation, Svenska Läkarsällskapet and from Svenska Livförsäkringsbolags namnd för Medicinsk Forskning.

REFERENCES

- ALPER, C.A., COLTEN, H.R., ROSEN, F.S., RABSON, A.R., MACNAB, G.M. & GEAR, J.S.S. (1972) Homozygous deficiency of C3 in a patient with repeated infections. *Lancet*, **ii**, 1179.
- AGNELLO, V. (1978) Complement deficiency states. *Medicine*, **57**, 1.
- DAVIS, C.A. & FORRISTAL, J. (1980) Partial properdin deficiency. *J. lab. clin. Med.* **96**, 633.

- DAVIS, C.A., VALLOTTA, E.H. & FORRISTAL, J. (1979) Serum complement levels in infancy: age related changes. *Pediat. Res.* 13, 1043.
- FEARON, D.T. & AUSTEN, K.F. (1980) The alternative pathway of complement—a system for host resistance to microbial infection. *N. Engl. J. Med.* 303, 259.
- GIANKOT, P.O. (1972) Crossed immunoelectrophoresis. *Scand. J. clin. lab. Invest.* 29, Suppl 124, 39.
- GOLDSCHNEIDER, I., GOTSCHLICH, E.C. & ARTENSTEIN, M.S. (1969) Human immunity to the meningococcus. I. The role of humoral antibodies. *J. exp. Med.* 129, 1307.
- GRUBB, A. (1970) Quantitation of immunoglobulin G by electrophoresis in agarose gel containing antibodies. *Scand. J. clin. lab. Invest.* 26, 249.
- GRUBB, A. (1974) Crossed immunoelectrophoresis and electroimmunoassay of IgM. *J. Immunol.* 112, 1420.
- GÖTZE, O., MEDICUS, R.G. & MÜLLER-EBERHARD, H.J. (1977) Alternative pathway of complement: nonenzymatic, reversible transition of precursor to active properdin. *J. Immunol.* 118, 525.
- LAURELL, A.-B., MÄRTENSSON, U. & SÖDERHOLM, A.G. (1977) Studies of C1 subcomponents in chronic urticaria and angioedema. *Int. Arch. Allergy appl. Immunol.* 54, 434.
- LAURELL, A.-B., MÄRTENSSON, U. & SÖDERHOLM, A.G. (1978) The development of simple tests for C1q, C1r, C1s, and C2 and the determination of properdin. In *Clinical aspects of the complement system* (ed. by W. Opferkuch, K. Rother & D. R. Schulz) p. 12. George Thieme Publishers, Stuttgart.
- MARTIN, A., LACHMANN, P.J., HALBWACHS, L. & HUBART, M.J. (1976) Hemolytic diffusion plate assays for factors B and D of the alternative pathway of complement activation. *Immunochimistry.* 13, 317.
- OLOFSSON, T., ODEBERG, H. & OLSSON, I. (1976) Granulocyte function in chronic granulocytic leukemia. II. Bactericidal capacity, phagocytic rate, oxygen consumption and granule protein composition in isolated granulocytes. *Blood*, 48, 581.
- PETERSEN, B.H., LEE, T.J., SNYDERMAN, R. & BROOKS, G.F. (1979) *Neisseria meningitidis* and *Neisseria gonorrhoeae* bacteremia associated with C6, C7 and C8 deficiency. *Ann. Intern. Med.* 90, 917.
- RAPP, H.J. & BURSOS, T. (1970) *Molecular basis of complement action*. Appleton-Century-Crofts, New York.
- ROBBINS, J.B. (1978) Vaccines for the prevention of encapsulated bacterial diseases: current status, problems and prospects for the future. *Immunochimistry.* 15, 839.
- SÖDERHOLM, A.G. (1975) Complement components in normal serum and plasma quantitated by electroimmunoassay. *Scand. J. Immunol.* 4, 25.
- SNYDERMAN, R., DURACK, D.T., MCCARTHY, G.A., WARD, F.E. & MEADOWS, L. (1979) Deficiency of the fifth component of complement in human subjects. Clinical, genetic and immunologic studies in a large kindred. *Am. J. Med.* 67, 638.
- STOSSEL, T.P., ALPER, C.A. & ROSEN, F.S. (1973) Serum dependent phagocytosis of paraffin oil emulsified with bacterial lipopolysaccharide. *J. exp. Med.* 137, 690.
- THOMPSON, R.A. & LACHMANN, P.J. (1977) A second case of human C3b inhibitor (KAF) deficiency. *Clin. exp. Immunol.* 27, 23.
- TRUBSSON, L., SÖDERHOLM, A.G. & LAURELL, A.-B. (1981) Screening of deficiencies in the classical and alternative pathways of complement by hemolysis in gel. *Acta path. microbiol. scand. Sect. C* 89, 161.
- WYATT, R.J., JULIAN, B.A. & GALLA, J.H. (1981) Properdin deficiency with IgA nephropathy. *N. Engl. J. Med.* 305, 1097.

Fulminant meningococcal infections in a family with inherited deficiency of properdin.

Jean H. Braconier, Sjöholm Anders G. and Söderström Claes

Running title. Fulminant meningococcal infection

Department of Infectious Diseases, University Hospital,
S-221 85 Lund and Department of Immunology, Institute of
Medical Microbiology, University of Lund, S-223 62 Lund,
Sweden

SUMMARY

Three males in a large kindred died of meningococcal infections. In the index patient, properdin (P) was not detectable in serum. Two healthy males with a selective P deficiency were found in the family. There was no general susceptibility to infections, nor to other diseases as suggested by the family history. The bactericidal activity for N. meningitidis group C, isolated from the index patient, was moderately reduced in P deficient serum, and was improved by addition of purified P.

INTRODUCTION

Acute meningococcal infections usually occur in individuals without protective antibodies (10). The importance of the complement system in resistance to N. meningitidis has been suggested by the observation of recurrent neisserial infections in patients with deficiencies of the terminal complement components C5, C6, C7, or C8 (16, 19, 22). The present study concerned the occurrence of lethal meningococcal infections in a family with an inherited deficiency of the alternative pathway component properdin (P). The index patient died of an infection with N. meningitidis group C. The bactericidal activity for the meningococci in P deficient serum was examined. Bactericidal antibodies to group A and C meningococci were measured before and after vaccination in several of the family members. The immunological investigation included determination of immunoglobulin levels and some aspects of cellular immunity. The P deficiency, and some of the impaired alternative pathway functions in P deficient serum have been documented elsewhere (21).

CASE REPORTS

Case 1 (H.M.). The index patient, a previously healthy 15-year-old boy was admitted to hospital in 1979 because of vomiting and fever. On arrival at hospital after 9 hours' disease he was in circulatory shock and his temperature was 41°C. Penicillin, gentamicin and corticosteroids were given. Treatment of acidosis resulted in a transient increase of the blood pressure. The boy, however, soon developed petechial bleedings and mental confusion. Artificial respiration was started and chloramphenicol given. The patient died 14 hours after the onset of initial symptoms. Haemoglobin was 122 g/l, blood leukocyte count $2.7 \times 10^9/l$ (60 % neutrophils), thrombocytes $100 \times 10^9/l$, fibrinogen normal, trombotest 13 % (normal > 50 %), activated partial thromboplastin time 70 s (normal < 35 s) and no increase of fibrinogen degradation products was found. Lumbar puncture disclosed clear fluid with a normal cell count, glucose and protein level. Cultures from blood and spinal fluid showed N. meningitidis group C. No autopsy was performed.

Case 2 (S.M.). A younger brother of H.M. had died several years earlier in a similar disease. S.M., a six-year-old boy, was sent to hospital in 1963 because of high fever, headache and finally confusion. On arrival at hospital, less than 20 hours after the debut of his illness, the boy was unconscious, in circulatory shock and developed multiple petechiae. He died within one hour, before other treatment than corticosteroids was given. Haemoglobin was 118 g/l, blood leukocyte count $14.2 \times 10^9/l$ (65 % neutrophils) and thrombocytes $66 \times 10^9/l$. Spinal fluid was clear with no red cells, four leukocytes/ μl , normal protein and chloride concentration. Necropsy revealed incipient meningitis, numerous petechiae in skin and in mucous membranes and hemorrhagic necrosis of the adrenals.

Culture from the nasopharynx before death showed N. meningitidis, as did culture from the base of the brain taken at the necropsy. β -hemolytic streptococci group A were also found in the nasopharynx before death and in the lungs at the necropsy.

Case 3 (S.L.). This 31-year-old man, a maternal uncle to H.M. and S.M., had earlier been healthy with the exception of a pneumonia three years previously. In 1959 he was admitted to hospital after about 12 hours' disease. He had high fever, multiple petechiae on the skin and was agitated. Examination of blood and spinal fluid was not possible because of his unrest. Penicillin, sulphonamide and corticosteroids were given, but the man died within 30 hours' disease. Cultures from spinal fluid and heart taken after death were negative. Necropsy showed purulent meningitis, purpuric lesions on the pericardium, in the peritoneal serosa and in the lungs. The adrenals were normal. The clinical diagnosis was meningococcal sepsis and meningitis.

Case 4 (I.I.). This boy, a maternal cousin of the brothers was one-year-old when taken to hospital because of high fever and vomittings. Lumbar puncture showed 2 red cells, 640 neutrophils and 530 lymphocytes/ μ l. Protein was slightly elevated and glucose level was normal. Blood leukocyte count was $13.8 \times 10^9/l$ (46 % neutrophils). Culture from spinal fluid was performed at a laboratory 400 km away and was negative. The boy was treated with chloramphenicol and made an uneventful recovery.

In 1974, at the age of 18, he was ill with high fever, headache and confusion. On arrival at hospital 30 hours later the condition had spontaneously improved and the patient was alert with low grade fever. A macular rash with central petechiae was found on the skin. There were no clinical signs of meningitis. Spinal fluid contained 5 red blood-cells, 5 neutrophils, 5 mononuclear cells/ μ l and normal

CASE REPORTS

Case 1 (H.M.). The index patient, a previously healthy 15-year-old boy was admitted to hospital in 1979 because of vomiting and fever. On arrival at hospital after 9 hours' disease he was in circulatory shock and his temperature was 41°C . Penicillin, gentamicin and corticosteroids were given. Treatment of acidosis resulted in a transient increase of the blood pressure. The boy, however, soon developed petechial bleedings and mental confusion. Artificial respiration was started and chloramphenicol given. The patient died 14 hours after the onset of initial symptoms. Haemoglobin was 122 g/l, blood leukocyte count $2.7 \times 10^9/\text{l}$ (60 % neutrophils), thrombocytes $100 \times 10^9/\text{l}$, fibrinogen normal, trombotest 13 % (normal > 50 %), activated partial tromboplastin time 70 s (normal < 35 s) and no increase of fibrinogen degradation products was found. Lumbar puncture disclosed clear fluid with a normal cell count, glucose and protein level. Cultures from blood and spinal fluid showed N. meningitidis group C. No autopsy was performed.

Case 2 (S.M.). A younger brother of H.M. had died several years earlier in a similar disease. S.M., a six-year-old boy, was sent to hospital in 1963 because of high fever, headache and finally confusion. On arrival at hospital, less than 20 hours after the debut of his illness, the boy was unconscious, in circulatory shock and developed multiple petechiae. He died within one hour, before other treatment than corticosteroids was given. Haemoglobin was 118 g/l, blood leukocyte count $14.2 \times 10^9/\text{l}$ (65 % neutrophils) and thrombocytes $66 \times 10^9/\text{l}$. Spinal fluid was clear with no red cells, four leukocytes/ μl , normal protein and chloride concentration. Necropsy revealed incipient meningitis, numerous petechiae in skin and in mucous membranes and hemorrhagic necrosis of the adrenals.

MATERIAL AND METHODS

Serum and EDTA plasma. Blood samples were obtained on one to three occasions from 34 members of the family in four generations. The sample from H.M. was obtained about 10 hours from the onset of disease. Serum and EDTA plasma were stored in aliquots at -80°C .

Plasma proteins. Complement components were quantified immunochemically by electroimmunoassay, and some functional assays were also performed (21). Complement component levels were given in % of the concentrations in a reference serum pool, said to contain the proteins at a concentration of 100 %. The levels of IgG, IgA and IgM were determined by electroimmunoassay (11, 12). IgE was measured by PRIST (Pharmacia Diagnostics AB, Uppsala, Sweden), and C-reactive protein by electroimmunoassay (15).

Coagulation studies. Activated partial thromboplastin time and prothrombin time (Thrombotest and Normotest, Nyegaard & Co AS, Oslo, Norway) were determined.

Specific antibodies. Bactericidal antibodies to group A and C meningococci were kindly determined by Dr. D. Danielsson, Örebro, Sweden, mainly as described by Frasci and Chapman (9). Assays for antibodies to tetanus toxoid and to Salmonella typhi and paratyphi B antigens (ELISA) were carried out at the National Swedish Bacteriological Laboratory (SBL), Stockholm, Sweden. Antibodies to morbilli and rubella virus, to streptolysin O and the titres of anti-A isoagglutinins in T.I. were determined by standard procedures.

Purified P. P in the native form was prepared essentially as described by Götze et al. (13), with omission of the final immune absorption step. The preparation was gel filtrated on Sepharose CL 6B and on Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala,

known contaminant.

Vaccines. Meningococcal polysaccharide vaccine serogroup A and C (Merieux, France) was given in a single dose. Booster injections were given to I.I. of tetanus toxoid, typhoid vaccine (TAB) and of inactivated polio virus types 1-3 (SBL).

Skin testing. Schick's reagent (SBL) and PPD (Statens Serum-institut, Copenhagen, Denmark) were used.

Leukocytes. White blood cell and differential counts were carried out by standard procedures. Tests for lymphocyte surface markers and lymphocyte stimulation with PHA were kindly performed by Dr. T. Hallberg, Lund (14). Granulocytes from I.I. and from two healthy controls were isolated with Isopaque-Ficoll (18).

Bacteria. N. meningitidis group C, as determined by Dr. D. Danielsson, Örebro, Sweden, were isolated from H.M. The strain was stored in foetal calf serum at -80°C , and cultivated on a solid medium (17). Before each experiment, the meningococci were grown over night in broth (22) at 37°C , and were then inoculated on fresh broth. After 4 hours at 37°C , the meningococci were harvested in the logarithmic growth phase and washed in phosphate buffered saline with 0.1 % gelatin. Viable bacteria accounted for 80-90 % of the organisms. The bacteria were adjusted to about 8×10^7 viable organisms per ml in veronal buffered saline with gelatin, Ca^{++} and Mg^{+++} (VBS) (20).

S. pneumoniae serotype 23F, obtained from Statens Serum-institut, Copenhagen, Denmark, were cultivated and used as described earlier (3).

Bactericidal activity of serum. Serum diluted in VBS was incubated during agitation at 37°C with N. meningitidis group C, 10^6 - 10^7 in a final volume of 0.25 ml. After 0 and 10 minutes, viable organisms were quantified by the pour plate method. All experiments were performed in duplicate. Bacterial killing was expressed in

% with the number of bacteria surviving in buffer alone as a reference. Pooled serum from 20 healthy donors and 6 individual normal sera were used as controls.

Granulocyte killing of *S. pneumoniae* serotyp 23F. About 1×10^6 granulocytes from T.T. and from two controls were incubated with approximately 5×10^6 pneumococci, in 8 % pooled control serum (3). Incubations were performed during rotation end-over-end. Viable bacteria were quantified at 0 and 30 minutes by colony counting using the pour plate method. Values were given in % of bacteria surviving in incubations without granulocytes.

RESULTS

Plasma proteins. P was undetectable ($< 2\%$ of the normal) in serum and EDTA plasma from H.M., and moderately low C1q and C2 values suggested classical pathway activation during the meningococcal infection. P was undetectable also in samples obtained from T.T. and L.L., who were healthy and showed normal or high levels of all other complement components (21). The immunoglobulin levels were normal in the three P deficient, except for a high IgE level in T.T. C-reactive protein was slightly increased in H.M. (Table I). Other family members were not P deficient. The parents of T.T. and H.M., and the father of L.L. showed normal P levels. Slightly decreased P concentrations were found in three female members of the family (21).

The function of the coagulation system was normal in L.L. and T.T. as assessed by activated partial thromboplastin time, Thrombotest and Normotest.

Specific antibodies. Bactericidal antibodies against meningococci were measured in 17 members of the family before and after vaccination with group A and C meningococcal polysaccharide. Before vaccination, antibodies to group A meningococci were detected in all, except T.T. with titers between 1:2 and 1:32. One month after vaccination the titers were increased by one or two titer steps in 11 and remained unchanged in six of the family members. In T.T. a titer of 1:64 was found one month after vaccination. Five months later the titer was 1:8. Tests for antibodies to group C meningococci were negative in 14 individuals, including T.T., and positive in four of the family before vaccination. After vaccination, antibodies to group C meningococci were present at comparatively low titers (1:1 to 1:4) in all but one.

On vaccination, T.I. gave a normal secondary antibody response to Tetanus toxoid and to Polio 1, 2, and 3. Antibodies to Salmonella B0 and D0 antigens did not increase after vaccination with TAB. T.I. showed antibodies to Rubella, but not to Morbilli virus. The anti-streptolysin was slightly raised. Isoagglutinins to blood group A were present at a titer of 1:32. Schick's test was negative.

Bactericidal activity of serum. The bacterial activity for N. meningitidis serogroup C was reduced in P deficient serum (L.L.) compared to the control sera. The sera were tested in a final concentration of 12.5, 25 and 50 % (Table II).

In a separate experiment, purified P was added to P deficient serum (L.L.) at a physiological concentration (about 100 % of the normal). The bactericidal activity of 50 % P deficient serum was examined with and without P added. After 10 minutes at 37°C, 60 % of the meningococci were killed in P deficient serum as compared with 82 % in the serum with P added and 85 % in the pooled control serum. Similar results were obtained with serum from T.I.

Lymphocytes. Lymphocyte surface markers and lymphocyte stimulation with PHA were studied in L.L., T.I., his mother and in the parents and in siblings to H.M. The proportions of B lymphocytes were normal as determined by detection of membrane immunoglobulin (3.0 - 9.5 %) and of membrane u chains (0.5 - 3.0 %).

Rosette tests using sheep erythrocytes for T lymphocytes were normal (56 - 71 %) as were tests detecting receptors for Fc of IgG (29 - 53 %) and complement (17 - 40 %). The lymphocytes responded in a normal fashion to stimulation with PHA. Skin tests with PPD for delayed type hypersensitivity in T.I. were negative at 2 T.U. but positive at 5 T.U.

Granulocyte killing of bacteria. The capacity of granulocytes from T.I. to phagocytize and kill S. pneumoniae type 23F was studied.

Granulocytes were incubated with pneumococci in pooled control serum in a final concentration of 8 %. The phagocytic killing after 30 minutes incubation did not differ significantly for the granulocytes from the patient (64% killed) and for the granulocytes from the two controls (72 and 75 % killed).

DISCUSSION

The incidence of meningococcal infections in Sweden is 100-200 cases per year (SBL). No epidemic outbreaks of meningococcal disease have occurred during the current years. With this background, the occurrence at different times of lethal meningococcal infections in three members of one family is remarkable, and might indicate a genetically determined susceptibility to meningococcal disease. A fourth member of the family also died of an acute infection, but the diagnosis was never established.

Resistance to meningococcal infections is known to be associated with acquisition of group specific antipolysaccharide antibodies that are bactericidal in the presence of complement (10).

The immunological investigations performed together with the family history argue against some of the immunodeficiency states that might be considered. We do not know if protective antibodies were present in the patients, who died of fulminant infections. In healthy members of the family bactericidal antibodies to groups A and C meningococci were detected before vaccination except in one male (T.I.), who was P deficient. T.I. and other members of the family gave an apparently normal antibody response to vaccination.

The index patient (H.M.), and two other male members of the family (T.I. and L.L.), demonstrated a complete deficiency of P in serum and EDTA plasma. P might possibly have been undetectable in H.M. due to complement activation, but T.I. and L.L. were healthy and showed normal or high levels of other complement components (21). The three P deficientes were related on the maternal side, and to our knowledge, the fathers were unrelated. Assuming an inherited deficiency, this would suggest X-linked transmission. Such a mode of inheritance would be consistent with possible P deficiency in the

three males (S.M., S.L. and S.E.J., who died earlier of fulminant infections. Since certain coagulation defects are known to be X-linked (4) it might be pointed out that bleeding abnormalities were not present in the family history, and were not found on investigation of the two healthy P deficienta. Partial P deficiency has been described by others, who suggested that the defect was inherited as an autosomal recessive trait (5, 26).

The present observations indicate that an inherited, complete deficiency of P may have predisposed for the fulminant infections in the family. P stabilizes the alternative pathway C3 convertase C3bBb, and can promote C3 activation initiated through the classical as well as through the alternative pathway (7). Thus, several functions dependent on efficient C3 activation may be impaired in P deficiency. Severe infections with a variety of bacteria, including N. meningitidis have been described in C3 deficiency syndromes (1, 2).

The index patient (H.M.) died of an infection with N. meningitidis group C. The polysaccharide capsule of group C meningococci contains sialic acid and the organisms might not be expected to activate the alternative pathway (7).

The bactericidal activity for the meningococci was only moderately decreased in sera from the healthy P deficienta, suggesting that the reaction was mainly dependent on antibody and the classical pathway. These findings can hardly explain the occurrence of fulminant meningococcal infections in P deficiency. In deficiencies of C6, C7 and C8, the very low serum bactericidal activity is thought to account for the susceptibility to neisserial infections (19). The comparatively mild course of disseminated meningococcal infections observed in these deficiency states could be due to a preserved capacity for opsonization (16). Low opsonic activity for S. pneumoniae (to be

published) and for endotoxin coated oil particles (21) has been demonstrated in P deficient serum.

It might be that resistance to infecting organisms such as N. meningitidis, is not particularly low in P deficiency. Endotoxin is released into the circulation in meningococcal disease, and is thought to play a prominent role in pathogenesis (6). A fulminant course of meningococcal infections in P deficiency might possibly be explained by an increased susceptibility to the actions of endotoxin.

Individuals with P deficiency should probably be immunized with meningococcal vaccine. Administration of plasma may be considered in the treatment of patients with severe infections and P deficiency. Repeated plasma infusions have previously been given in two patients with a C3 deficiency syndrome (2, 25).

It has been suggested that analysis of complement is indicated in patients with recurrent neisserial infections and may be warranted in patients with first episodes (19). Concluding from the present findings, such analysis should include evaluation of alternative pathway as well as of classical pathway function. This can be accomplished by simple screening assays (8, 24).

REFERENCES

1. Agnello V.: Complement deficiency states. *Medicine* 57:1, 1978.
2. Alper, C.A., Abramson, N., Johnston, R.B., Jr., Jandl, J.H. & Rosen, F.: Studies in vivo and in vitro on an abnormality in the metabolism of C3 in a patient with increased susceptibility to infection. *J Clin Invest* 49:1975, 1970.
3. Braconier, J.H. & Odeberg, H.: Granulocyte phagocytosis and killing of virulent and avirulent serotypes of *Streptococcus pneumoniae*. *J Lab Clin Med* 100:279, 1982.
4. Davie, E.W. and Hanahan, D.J.: Blood coagulation proteins. In: *The plasma proteins*, p. 421 (ed. F.W. Putnam). Academic Press Inc. New York 1977.
5. Davis, C.A. & Forristal, J.: Partial properdin deficiency. *J Lab Clin Med* 96:633, 1980.
6. DeVoe, I.W.: The interaction of polymorphonuclear leukocytes and endotoxin in meningococcal disease: a short review. *Canad J Microbiol* 126:729, 1980.
7. Fearon, D.T. & Austen, K.F.: The alternative pathway of complement - a system for host resistance to microbial infection. *N Engl J Med* 303:259, 1980.
8. Fong, J.S.C. & Renaud, L.: A hemolytic plate method for alternative-pathway complement activity assay. *Am J Clin Path* 69:156, 1978.
9. Frasch, C.E. & Chapman, S.S. Classification of *Neisseria meningitidis* group B into distinct serotypes. I. Serological typing by a microbactericidal method. *Infect Immun* 5:98, 1972.
10. Goldschneider, I., Gotschlich E.C. & Artenstern, M.S.: Human immunity to the meningococcus. I. The role of humoral antibodies. *J Exp Med* 129:1307, 1969.

11. Grubb, A.: Quantitation of immunoglobulin G by electrophoresis in agarose gel containing antibodies. *Scand J Clin Lab Invest* 26: 249, 1970.
12. Grubb, A.: Crossed immunoelectrophoresis and electroimmunoassay of IgM. *J Immunol* 112:1420, 1974.
13. Götze, O., Medicus, R.G. & Müller-Eberhard, H.J.: Alternative pathway of complement: non-enzymatic, reversible transition of precursor to active properdin. *J Immunol* 118:525, 1977.
14. Hallberg, A., Hallberg, T. & Holmberg, L. PPD testing as a diagnostic aid in non-tuberculous mycobacteriosis. *Acta Paediatr Scand* 69:511, 1980.
15. Johnsson, U. & Prellner, K.: Purification of C-reactive protein on DEAE-cellulose by a simple two-step procedure utilizing the calcium-dependency of the protein. *Biochim Biophys Acta* 495: 349, 1977.
16. Matthews, N., Stark, J.M., Harper, P.S., Doran, J. & Jones, D.M.: Recurrent meningococcal infections associated with a functional deficiency of the C8 component of human complement. *Clin Exp Immunol* 39:53, 1980.
17. Mårdh, P.A., Mårtensson, D. & Soltész, L.V.: An effective, simplified medium for the culture of *Neisseria gonorrhoeae*. *Sex Transm Dis* 5:10, 1978.
18. Olofsson, T., Odeberg, H., Olsson, I.: Granulocyte function in chronic granulocytic leukemia. II. Bactericidal capacity, phagocytic rate, oxygen consumption and granule protein composition in isolated granulocytes. *Blood* 48:581, 1976.
19. Petersen, B.H., Lee, T.J., Snyderman, R. & Brooks, G.F.: Neisseria meningitidis and Neisseria gonorrhoeae bacteremia associated with C6, C7, or C8 deficiency. *Ann Intern Med* 90:917, 1979.

20. Rapp, H.J. & Borsos, T.: Molecular basis of complement action, p. 77. Appleton-Century-Crofts. New York 1970.
21. Sjöholm, A.G., Braconier, J.H. & Söderström, C.: Properdin deficiency in a family with fulminant meningococcal infections. Clin Exp Immunol. In press.
22. Snyderman, R., Durack, D.T., McCarthy, G.A., Ward, F.E. & Meadows, L.: Deficiency of the fifth component of complement in human subjects. Clinical, genetic and immunologic studies in a large kindred. Am J Med 67:638, 1979.
23. Soltész, L.V. & Mårdh, P.A.: Serum-free liquid medium for Neisseria Gonorrhoeae. Current Microbiol 4:45, 1980.
24. Truedsson, L., Sjöholm, A.G. & Laurell, A.-B.: Screening for deficiencies in the classical and alternative pathways of complement by hemolysis in gel. Acta Path Microbiol Scand. Sect. C. 89:161, 1981.
25. Wahn, V., Rother, U., Rauterberg, E.W., Day, N.K. & Laurell, A.B.: C3b inactivator deficiency: Association with an alpha-migrating factor H. J Clin Immunol 1:228, 1981.
26. Wyatt, R.J., Julian, B.A. & Galla, J.H.: Properdin deficiency with IgA nephropathy. N Engl J Med 305:1097, 1981.

Acknowledgements

This investigation was supported by the Swedish Medical Research Council (B 80-17X-05430-02 and B 83-16X-68), by the Medical Faculty of Lund, by the foundations of Österlund, Segerfalk, Forssman and Kock.

The technical assistance of Mrs Asta Lundquist and Mrs Britt Turesson is greatly appreciated.

Table I. Immunoglobulins, C-reactive protein (CRP) and white blood count (WBC) in the three properdin deficient individuals

	H.M.	T.T.	L.L.	Reference area
IgG	7.7	12.7	6.9	6.5-15.0 g/l
IgM	0.5	1.5	1.3	0.4-2.0 g/l
IgA	0.5	1.2	1.6	0.5-3.0 g/l
IgE	45	4360	1.6	<100 kU/l
CRP	20	< 12	<12	<12 mg/l
WBC (% poly)	2.7 (63%)	7.6 (65%)	6.7 (51%)	4-10 x 10 ⁹ /l (50-75%)

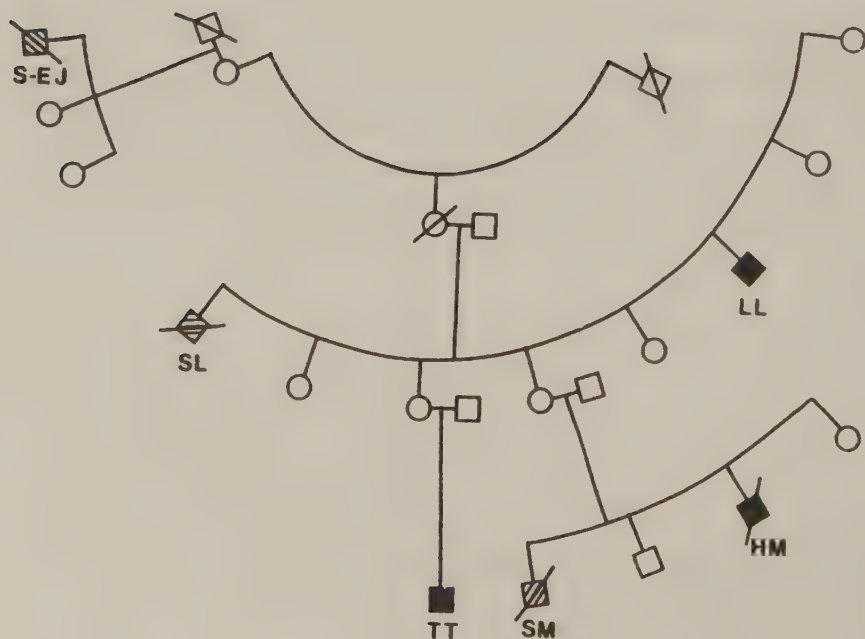
Table II. Bactericidal activity of P deficient serum (PDs) and pooled and individual control sera (NHs) against *N. meningitidis* serogroup C. About 2 x 10⁶ organisms were incubated with serum at 37°C for 10 minutes. Bacterial killing was expressed in %, using the number of organisms surviving in buffer alone as a reference.

	Serum concentration		
	12.5 %	25 %	50 %
PDs	58	56	75 ¹
Pooled NHs	71	82	97 ¹
Individual NHs	-	-	97 (96-99) ²

¹ Mean of two separate experiments

² Range, n=6

Figure Family pedigree. Males are represented by squares and females by circles. Deceased family members are indicated by an oblique line across the symbol. Closed symbols denote properdin deficient individuals. Hatched symbols are used for patients with lethal infections and unknown properdin concentrations. Individuals referred to in the case reports are indicated by their initials. H.M. was the properdin deficient index patient.



Granulocyte phagocytosis of Streptococcus pneumoniae in properdin deficient serum.

Running title: Properdin and pneumococcal opsonins

Braconier, Jean H.^{1*}, Odeberg, Håkan² and Sjöholm, Anders G.³.

¹Department of Infectious Diseases, University Hospital, S-221 85 Lund, ²Research Department 2, E block, University Hospital, S-221 85 Lund, ³Department of Immunology, Institute of Medical Microbiology, Sölvegatan 23, S-223 63 Lund, Sweden

Correspondence to: Dr. J.H. Braconier

Department of Infectious Diseases,
University Hospital,
S-221 85 Lund, Sweden
Telephone: 046-101129

SUMMARY

Properdin (P) deficient human serum containing type specific anticapsular antibodies, and with an intact classical pathway of complement, did not support efficient granulocyte phagocytosis of serotype 6A, 14, 19F, 23F or 35 S. pneumoniae in an opsonophagocytic assay. Compared with pooled control serum, the difference was most pronounced for serotypes 14 and 23F, and at a final serum concentration of 16 %. The reduced phagocytic killing of serotype 23F S. pneumoniae in P deficient serum was due rather to defective opsonization than to impaired intracellular killing. The uptake of C3 by the serotype 23 strain, was found to be low in P deficient serum. Addition of native P promoted C3 fixation as well as opsonization. The activity of P was abolished by heat treatment (56°C, 30 minutes). Experiments with MgEGTA to block C1 activation, suggested that serotype 23F pneumococci were more dependent on the classical pathway for opsonization than were the serotype 35 pneumococci, that appear to be partly opsonized through the alternative pathway alone.

INTRODUCTION

The importance of type specific anticapsular antibodies and complement for opsonization of S. pneumoniae is well established (29). It is also known that phagocytes possess receptors for IgG-Fc and for C3b, the activated form of C3 (7). The main role of antibody in opsonization of S. pneumoniae is probably to mediate the fixation of opsonic C3b to the bacterial surface by classical, and possibly also by alternative pathway activation of complement (3, 12). Many serotypes of S. pneumoniae activate the alternative pathway (10, 23, 26), which obviously contributes to opsonization (12, 13, 17). At least some serotypes are capable of fixing opsonic C3b by classical pathway activation through C-reactive protein (6). In addition, humoral factors may stimulate intracellular killing of bacteria (14, 16).

In attempts to investigate the relative roles of the two complement pathways for opsonization of different serotypes of S. pneumoniae, studies have been performed with serum reagents providing a nonfunctional classical pathway (12, 13, 17). The opsonic requirements for type 14 S. pneumoniae have been examined with sera depleted of factor D (12). Not much is known, however, of the influence on opsonization of an impaired alternative pathway.

A selective deficiency of properdin (P) was recently described in two healthy males belonging to a family with an apparent susceptibility to fulminant meningococcal infections (25). In the present study, P deficient serum was used to study the influence of properdin and the alternative pathway on granulocyte phagocytosis of S. pneumoniae. Five serotypes (6A, 14, 19f, 23F and 35) of S. pneumoniae were examined.

MATERIALS AND METHODS

Serum. Normal sera were obtained from 20 healthy adults. The sera were kept individually or in a serum pool, aliquots being stored at -80°C . P deficient serum was collected from two apparently healthy adults (T.T. and L.L.). T.T. was the cousin, and L.L. the uncle of a properdin deficient boy, who had died of a fulminant meningococcal infection. In the P deficient (<0.6 mg per l) sera, described elsewhere in more detail, (25), the concentrations of C1q, C1r, C1s, C4, C2, C3, C5, C6, C7, C8, C $\bar{1}$ inactivator, C4 binding protein and the factors B, D, I and H were normal or moderately high, and C9 was present. The sera supported immune hemolysis, but alternative pathway functions, such as the activation of C3 by zymosan or inulin and the opsonization of endotoxin-coated oil particles, were grossly impaired. The levels of IgG, IgA and IgM were normal, and no C-reactive protein was detected (<12 mg per l). Since the L.L. serum showed moderately high levels of the C1 subcomponents and of C3 (149-183 % of normal), it was only used in some experiments.

Native P. P was purified according to Götze et al (1977) but omitting the final immune absorption step. Gel filtration was done on Sepharose CL-6B and on Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden). Assuming P at 27.5 mg/l (5) in the pooled serum used for reference, the preparations contained P at 116 and 160 mg per l as determined by electro-immunoassay (25). Small amounts of IgG were the only known contaminant. The functional state of P was defined by its capacity to restore alternative pathway functions in P deficient serum, without causing more C3 cleavage than occurs in normal sera in the absence of activators (15, 25).

Blocking of C1 activation. In some experiments, sera were chelated with ethylene glycol tetra acetate (EGTA) at 10 mmol/l, and magnesium (Mg) at 2.5 mmol/l (9).

Crossed immunoelectrophoresis. (11) was used to study the cleavage of C3 in MgEGTA chelated serum, following incubation for 30 minutes with zymosan (Koch-Light laboratories, Ltd, England) at 2 mg per ml (25).

Type specific anticapsular antibodies. Antibodies against pneumococcal polysaccharide were determined using ELISA, kindly performed by Dr. J. Henriksen, Statens Seruminstitut, Copenhagen, Denmark (22). Antibodies to serotype 6A, 14, 19F and 23F *S. pneumoniae* were present in the P deficient sera of I.I. and L.L., and in the pooled control serum, with values similar to those found in healthy adults.

Bacteria. *S. pneumoniae* serotypes 6A, 14, 19F, 23F and 35 were obtained from Statens Seruminstitut, Copenhagen and the Institute of Medical Microbiology, Lund, Sweden. The stock cultures were kept at -80°C in fetal calf serum. Before each experiment the pneumococci were cultivated anaerobically (GasPak, Becton, Dickinson and Company, Cockeysville, Md. USA) over night on blood agar. The bacteria were then inoculated on Brain Heart Infusion broth (Difco) supplied with 50 mg per ml cysteine chlorid and cultured at 37°C in an anaerobic milieu. The pneumococci were harvested in the logarithmic growth phase, washed twice, and suspended in Hanks' balanced salt solution (HBSS) buffered with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), pH 7.4.

Granulocytes. Granulocytes from healthy individuals were isolated using the Isopaque-Ficoll system of Böyum (4). After lysis of red cells with 0.87 % NH_4Cl , the granulocytes were washed twice and suspended in HEPES-HBSS as described previously (21).

Opsonization of *S. pneumoniae*. Opsonization was measured as the phagocytic killing of pneumococci by granulocytes. Intragranulocytic killing of pneumococci has been shown to be rapid in the assay system (2). *S. pneumoniae* $5-10 \times 10^6$ were incubated, in various concentrations of serum in HEPES-HBSS, for 5 min at 37°C before addition of 10^6 granulocytes. Incubation was then continued at 37°C during end-over-end rotation in a total volume of 0.5 ml. Aliquots of 20 μl were removed at 0 and 30 min, and diluted in 8 ml distilled water containing 0.05 % bovine serum albumin. Viable bacteria were determined by colony counting, using the pour plate method. The results were expressed in % of the bacteria surviving at 30 minutes in the serum, tested without granulocytes. All experiments were performed in duplicate. When bacterial killing was in the range between 20-85 % of that in the control, the random error of the method was 5.9 % (SD).

Viable bacteria associated with granulocytes were separated by differential centrifugation (28) at 4°C , $160 \times g$, 5 min, after the addition of 2.5 ml cold HEPES-HBSS. The granulocytes were washed twice, and the number of viable bacteria in the cell pellet was determined.

Fixation of C3. Anti-C3d rabbit IgG (Anti-human C3d complement, DAKOPATTS a/s, Copenhagen, Denmark) was labeled with ^{125}I (IMS 3.30 100 mCi/ml, Amersham, Buckinghamshire, England) using the chloramine-I method (18). S. pneumoniae serotype 23F, 10^6 , were incubated with 16 % serum in HEPES-HBSS at 37°C in a total volume of 100 μl . After 0, 5, 10 and 20 minutes, 20 μl labeled anti-C3d (2×10^7 cpm) was added in 4 ml HEPES-HBSS. After 15 minutes at 37°C with the labeled antibody, the bacteria were washed twice in HEPES-HBSS. The radioactivity in the bacterial cell pellet was measured (Gamma Sample Counter, LKB, Stockholm, Sweden).

RESULTS

Phagocytic killing of *S. pneumoniae* in P deficient serum.

The phagocytic killing of *S. pneumoniae* serotypes 6A, 14, 19F, 23F and 35 was studied in 8, 16 and 32 % P deficient serum (I.I.) and in pooled control serum. All serotypes were inefficiently killed in the presence of P deficient serum except for 6A and 35 at high serum concentrations (Fig. 1). The opsonic requirements of the serotypes 6A and 23F were also investigated, using six individual control sera. The opsonic activity of the control sera was similar to that of the pooled control serum, and gave more efficient phagocytic killing than P deficient serum. Most of the subsequent experiments were performed with the serotype 23F *S. pneumoniae*. In one experiment, the phagocytic killing of this strain was examined in two P deficient sera (I.I. and L.L.) and in pooled control serum at serum concentrations of 32 %. In the presence of pooled control serum, 10 % of the serotype 23F pneumococci survived after 30 minutes, as compared with 68 % and 31 % in the sera of I.I. and L.L., respectively.

An attempt was made to distinguish between ingestion and intracellular killing of serotype 23F *S. pneumoniae* in P deficient serum. The pneumococci were incubated with granulocytes and P deficient serum (I.I.), or pooled control serum, at serum concentrations of 8 and 32 %. After differential centrifugation, the number of viable, granulocyte associated pneumococci, comprising attached and ingested organisms, was found to be low, both when the pneumococci had been incubated in P deficient serum, and in the control serum (Table I). Findings were consistent with efficient intracellular killing following ingestion, also in P deficient serum.

Restitution experiments. The opsonic activity for serotype 23F S. pneumoniae in 16 % P deficient serum, was examined with the addition of native P. P was added to the incubation mixtures at 4.6, 0.9 and 0.2 mg/l, corresponding to concentrations in serum of about 100, 20 and 5 % of normal. Opsonization was slightly improved by addition of P at 5 %. When P was added at 20 % and above, the opsonic activity of P deficient serum was found to be similar to that of the pooled control serum (Fig. 2). The opsonic activity of the control serum was not appreciably affected by addition of P.

The opsonic activity in 8 % P deficient serum was gradually increased by the addition of pooled control serum at final concentrations of 0.5, 1, 2 and 4 %. In contrast, heat inactivated pooled control serum (56°C, 30 minutes), added at the same concentrations, did not promote opsonization. These findings provided further argument against lack of heat stable opsonins, such as specific antibody, in P deficient serum, but were surprising since P has been reported to resist treatment at 56°C (19). P was subjected to heat treatment (56°C, 30 minutes), either alone in buffer (160 mg/l, 0.1 M Tris, 0.4 M NaCl, pH 7.4) or in P deficient serum at normal concentration (26.7 mg/l). The heat treated preparations did not promote opsonization in 16 % P deficient serum. Parallel control experiments showed that untreated P restored the opsonic activity in P deficient serum, even when added together with heat inactivated pool control serum, or with heat inactivated P deficient serum. Furthermore, heat treated native P in three preparations, did not promote C3 cleavage in P deficient serum incubated with zymosan.

Phagocytic killing of serotypes 23F and 35 S. pneumoniae in MgEGTA chelated serum. When C1 activation was blocked with MgEGTA, the opsonic activity for serotype 23F S. pneumoniae was low, both in P deficient serum

and in pooled control serum (Fig. 3 left). Chelation with MgEGTA resulted in a more pronounced decrease of the opsonic activity for serotype 35 S. pneumoniae in P deficient serum, than in the control serum (Fig. 3 right).

Fixation of C3 by serotype 23F S. pneumoniae in P deficient serum.

The uptake of C3 by the pneumococci on incubation in P deficient serum or pooled control serum, was assessed with ^{125}I -labeled anti-C3d.

The pneumococci fixed less C3 in 16 % P deficient serum, than in control serum. When P was added at 4.6 mg/l, the uptake by the pneumococci of C3 in P deficient serum became similar to that in control serum (Fig. 4).

DISCUSSION

In this study we used the sera from two healthy adults with a selective deficiency of P (25). The sera contained anticapsular antibodies to the pathogenic pneumococci tested, and provided a reagent with an intact classical pathway of complement, but with impaired alternative pathway functions.

The granulocyte opsonophagocytic killing of S. pneumoniae serotypes 6A, 14, 19F, 23F and 35 was less efficient in P deficient serum than in control serum. Judging from the low recovery of viable serotype 23 pneumococci associated with the granulocytes at the end of incubation, the reduced phagocytic killing in P deficient serum was due rather to defective opsonization than to impaired intracellular killing. On the other hand, efficient killing of ingested bacteria by monocytes appears to require extracellular complement with a functional alternative pathway (16). Such a mechanism might have contributed to our findings.

The opsonic activity of P deficient serum for serotype 23 pneumococci was restored to normal by the addition of fairly low amounts native P. This is consistent with the earlier observation of unimpaired opsonic activity for pneumococci in partially P deficient serum (5).

Restoration experiments with fresh and heat inactivated control serum suggested that the activity of P might be heat labile. This is supported by the fact that heat treatment (56°C, 30 minutes) of the native P used, destroyed its capacity to promote either the activation of C3 by zymosan or the opsonization of pneumococci in P deficient serum. Minta & Vetvutana-pibul (1978) reported that P in the activated form retained its activity in hemolytic assay after heat treatment at 56°C. Differences in the P preparations, or in the assays used, might explain these conflicting findings.

For some bacteria, the efficiency of opsonization varies with the amount of surface fixed C3, following incubation in serum (27). In the present study, efficient uptake of C3 by serotype 23 S. pneumoniae was shown to be dependent on P, which could explain the low opsonic activity of P deficient serum for these organisms.

The site at which the opsonic C3 was fixed is not clear. Cell walls, even of encapsulated pneumococci, account for most of the C3 fixation through the alternative pathway in serum (30, 31). However, recent evidence suggests that cell wall-bound C3b is not necessarily opsonic, since capsular polysaccharide can prevent its interaction with C3b receptors (3). Interestingly, experiments with MgEGTA to block C1 activation indicated that serotype 23F S. pneumoniae were poorly opsonized through the alternative pathway alone. The marked influence of the alternative pathway in unchelated serum might be due to amplification secondary to limited C3b activation, mediated by anticapsular antibody and the classical pathway (8). Opsonization of the serotype 35 pneumococci, that may be considered fairly apathogenic (2), appeared to be more independent of the classical pathway.

Pneumococcal and other bacterial infections are common in patients with hypogammaglobulinemia (24) and in C3 deficiency syndromes (1). Repeated pneumococcal infections have been reported in two patients with C2 deficiency and low concentrations of factor B (20), but are not usually encountered in deficiencies of the classical pathway components (1). These clinical associations could be related to the opsonic requirements of S. pneumoniae. In the family with properdin deficiency studied, there was no evidence of increased susceptibility to pneumococcal infections (25). It is possible that an impaired function of the alternative pathway becomes decisive in resistance to S. pneumoniae, only when other opsonization defects are also present.

REFERENCES

1. Agnello, V. 1978. Complement deficiency states. *Medicine* 57:1-23.
2. Braconier, J.H., and H. Odeberg. 1982. Granulocyte phagocytosis and killing of virulent and avirulent serotypes of Streptococcus pneumoniae. *J. Lab. clin. Med.* 100:279-287.
3. Brown, E.J., S.W. Hosea, C.H. Hammer, C.G. Burch, and M.M. Frank. 1982. A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. *J. clin. Invest.* 69:85-98.
4. Böyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. clin. Lab. Invest.* 21, suppl. 97.
5. Davis, C.A., and J. Forristal. 1980. Partial properdin deficiency. *J. Lab. clin. Med.* 96:633-639.
6. Edwards, K.M., H. Gewurz, T.F. Lint, and C. Mold. 1982. A role for C-reactive protein in the complement-mediated stimulation of human neutrophils by type 27 Streptococcus pneumoniae. *J. Immunol.* 128:2493-2496.
7. Ehlenberger, A.G., and V. Nussenzweig. 1977. The role of membrane receptors for C3b and C3d in phagocytosis. *J. exp. Med* 145:357-371.
8. Fearon, D.T., and K.F. Austen. 1980. The alternative pathway of complement - a system for host resistance to microbial infection. *N. Engl. J. Med.* 303:259-263.
9. Fine, D.P., S.R. Marney, Jr., D.G. Colley, J.S. Sergent, and R.M. Des Prez. 1972. C3 shunt activation in human serum chelated with EGTA. *J. Immunol.* 109:807-809.
10. Fine, D.P. 1975. Pneumococcal type-associated variability in alternate complement pathway activation. *Infect. Immun.* 12:772-778.
11. Ganrot, P.O. 1972. Crossed immunoelectrophoresis. *Scand. J. clin. Lab. Invest.* 29, suppl. 124:39-47.

12. Gardner, S.E., D.C. Anderson, B.J. Webb, A.E. Stitzel, M.S. Edwards, R.E. Spitzer, and C.J. Baker. 1982. Evaluation of Streptococcus pneumoniae type XIV opsonins by phagocytosis-associated chemiluminescence and a bactericidal assay. *Infect. Immun.* 35:800-808.
13. Giebink, G.S., J. Verhoef, P.K. Peterson, and P.G. Quie. 1977. Opsonic requirements for phagocytosis of Streptococcus pneumoniae types VI, XVIII, XXIII, and XXV. *Infect. Immun.* 18:291-297.
14. Guckian, J.C., G.D. Christensen, J.E. Schweinle, and D.P. Fine. 1981. Opsonization of pneumococci. I. Heat-labile serum activity other than complement is required for killing by human polymorphonuclear leukocytes. *J. Immunol.* 127:1659-1665.
15. Götze, O., R.G. Medicus, and H.J. Müller-Eberhard. 1977. Alternative pathway of complement: nonenzymatic, reversible transition of precursor to active properdin. *J. Immunol.* 118:525-528.
16. Leijh, P.C.J., M.T. van der Barselaar, T.L. van Zwet, M.R. Daha, and R. van Furth. 1979. Requirement of extracellular complement and immunoglobulin for intracellular killing of micro-organisms by human monocytes. *J. clin. Invest.* 63:772-784.
17. Matthay, K.K., W.C. Mentzer, D.W. Wara, H.K. Preisler, N.B. Lameris, and A.J. Ammann. 1981. Evaluation of the opsonic requirements for phagocytosis of Streptococcus pneumoniae serotypes VII, XIV, and XIX by chemiluminescence assay. *Infect. Immun.* 31:228-235.
18. McCahey, P.J., and F.J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Archs Allergy* 29:185-189.
19. Minta, J.O., and W. Vetvutanapibul. 1978. The effects of heat, pH and chemical agents on the functional activity of properdin. *Immunochemistry* 15:93-96.
20. Newman, S.L., L.B. Vogler, R.D. Feigin, and R.B. Johnston. 1978. Recurrent septicemia associated with congenital deficiency of C2 and partial deficiency of factor B and the alternative complement pathway. *N. Engl. J. Med.* 299:290-292.

21. Odeberg, H., T. Olofsson, and I. Olsson. 1975: Granulocyte function in chronic granulocytic leukemia. I. Bactericidal and metabolic capabilities during phagocytosis in isolated granulocytes. *Br. J. Haematol.* 29:427-441.
22. Pedersen, F.K., and J. Henrichsen. 1982. Detection of antibodies to pneumococcal capsular polysaccharides by enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* 15:372-378.
23. Prellner, K. 1979. Complement activation by pneumococci associated with acute otitis media. *Acta. path. microbiol. scand. Sect. C*, 87: 213-216.
24. Rosen, F.S., and C.A. Janeway. 1966. The gammaglobulins. III. The antibody deficiency syndromes. *N. Engl. J. Med.* 275:709-715.
25. Sjöholm, A.G., J.-H. Braconier, and C. Söderström. 1982. Properdin deficiency in a family with fulminant meningococcal infections. *Clin. exp. Immunol.* 50. In press.
26. Stephens, C.G., R.C. Williams, Jr., and W.P. Reed. 1977. Classical and alternative complement pathway activation by pneumococci. *Infect. Immun.* 17:296-302.
27. Verbrugh, H.A., W.C. van Dijk, M.E. van Erne, R. Peters, P.K. Peterson, and J. Verhoef. 1979. Quantitation of the third component of human complement attached to the surface of opsonized bacteria: opsonin-deficient sera and phagocytosis-resistant strains. *Infect. Immun.* 26:808-812.
28. Verhoef, J., P.K. Peterson, and P.G. Quie. 1977. Kinetics of staphylococcal opsonization, attachment, ingestion and killing by human polymorphonuclear leukocytes: a quantitative assay using ^3H -thymidine labeled bacteria. *J. Immunol. Methods* 14:303-311.
29. Winkelstein, J.A. 1973. Opsonins: their function, identity and clinical significance. *J. Pediatr.* 82:747-753.

30. Winkelstein, J.A., J.A. Bocchini, Jr., and G. Schiffman. 1976.

The role of the capsular polysaccharide in the activation of the alternative pathway by the pneumococcus. *J. Immunol.* 116:367-370.

31. Winkelstein, J.A., A.S. Abramovitz, and A. Tomasz. 1980. Activation

of C3 via the alternative complement pathway results in fixation of C3b to the pneumococcal cell wall. *J. Immunol.* 124:2502-2506.

ACKNOWLEDGEMENTS

The investigation was supported by the Swedish Medical Research Council (B81-17X-05430-02 and B83-16X-68), the Medical faculty of Lund, the foundations of Österlund, Segerfalk and Forssman and by Svenska livförsäkringsbolags nämnd för medicinsk forskning. The technical assistance of Mrs Asta Lundquist and Mrs Britt Thuresson is greatly appreciated.

Legends to figures

Figure 1. Phagocytic killing S. pneumoniae (serotypes 6A, 14, 19F, 23F and 35) in control serum (hatched columns) and in properdin deficient serum (open columns). 10^6 granulocytes were incubated with $5-10 \times 10^6$ pneumococci in 8, 16 and 32 % serum. Viable bacteria (mean of duplicate or triplicate experiments) were quantified after 30 minutes at 37°C, and given in % of the bacteria surviving in serum alone.

Figure 2. Phagocytic killing of serotype 23F S. pneumonia in 16 % properdin deficient serum (PD), with addition of purified properdin (P). With respect to serum, P was added in concentrations corresponding to 5, 20 and 100 % of the normal. Experimental conditions were similar to those given in figure 1. NHS denotes pooled control serum.

Figure 3. Opsonic activity for serotype 23F (left) and 35 (right) S. pneumoniae in properdin deficient serum (PDs) and pooled control serum (NHS). Influence of chelation with MgEGTA ($Mg^{++} 2.5 \text{ mmol/l}$, EGTA 10 mmol/l). Other experimental conditions were similar to those given in figure 1.

Figure 4. Uptake of ^{125}I -labeled anti-C3d by serotype 23F S. pneumoniae incubated in properdin deficient serum (PDs) and in pooled control serum (NHS). The bacteria were incubated at 37°C in 16 % serum for 0, 5, 10 or 20 minutes before addition of ^{125}I -labeled anti-C3d. Purified properdin (P) was added to PDs in the incubation mixture at 4.6 mg/l corresponding to a physiological P concentration in the diluted serum.

Table 1

Viable, granulocyte associated S. pneumoniae serotype 23f quantified after differential centrifugation, compared with the total number of surviving pneumococci in 8 and 32 % P deficient (PDs) and in pooled control serum (NHs). Incubation was carried out at 37°C for 30 min^{a)}.

Opsonin source	Total number surviving bacteria (% of buffer control)	Viable, granulocyte associated bacteria (% of total number surviving bacteria)
Buffer control	1.03×10^7	-
PDs 8 %	1.02×10^7 (99%)	4.0×10^4 (0.4 %)
PDs 32 %	0.45×10^7 (44%)	6.0×10^4 (1.3 %)
NHs 8%	0.34×10^7 (33%)	5.4×10^4 (1.6 %)
NHs 32 %	0.19×10^7 (18%)	15.6×10^4 (8.2 %)

a) Bacteria: granulocyte ratio 10:1

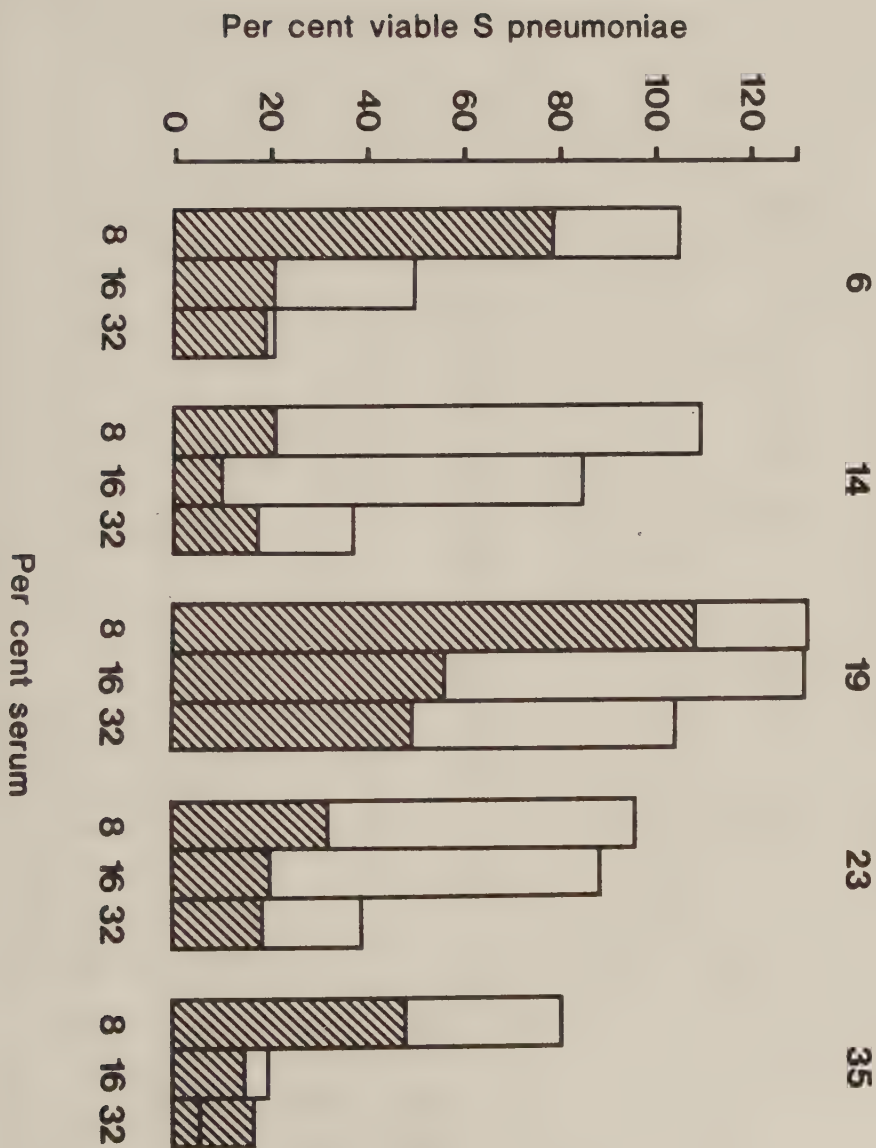


Figure 1

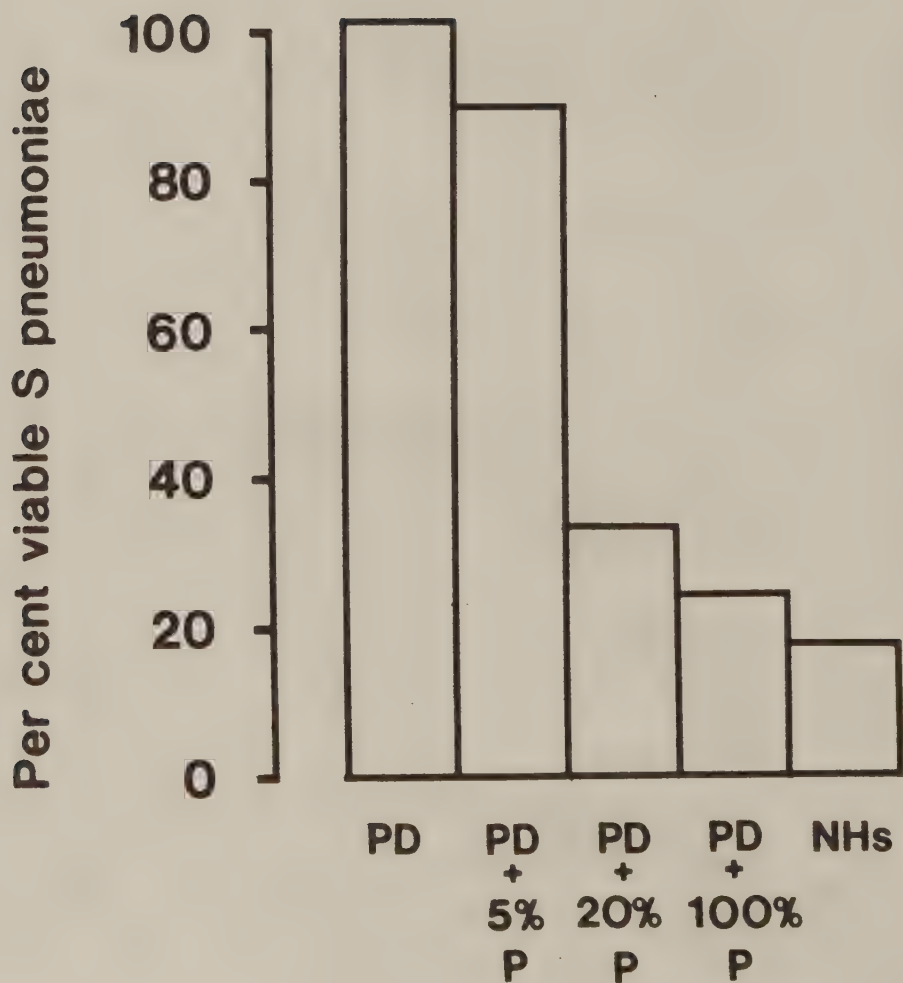


Figure 2

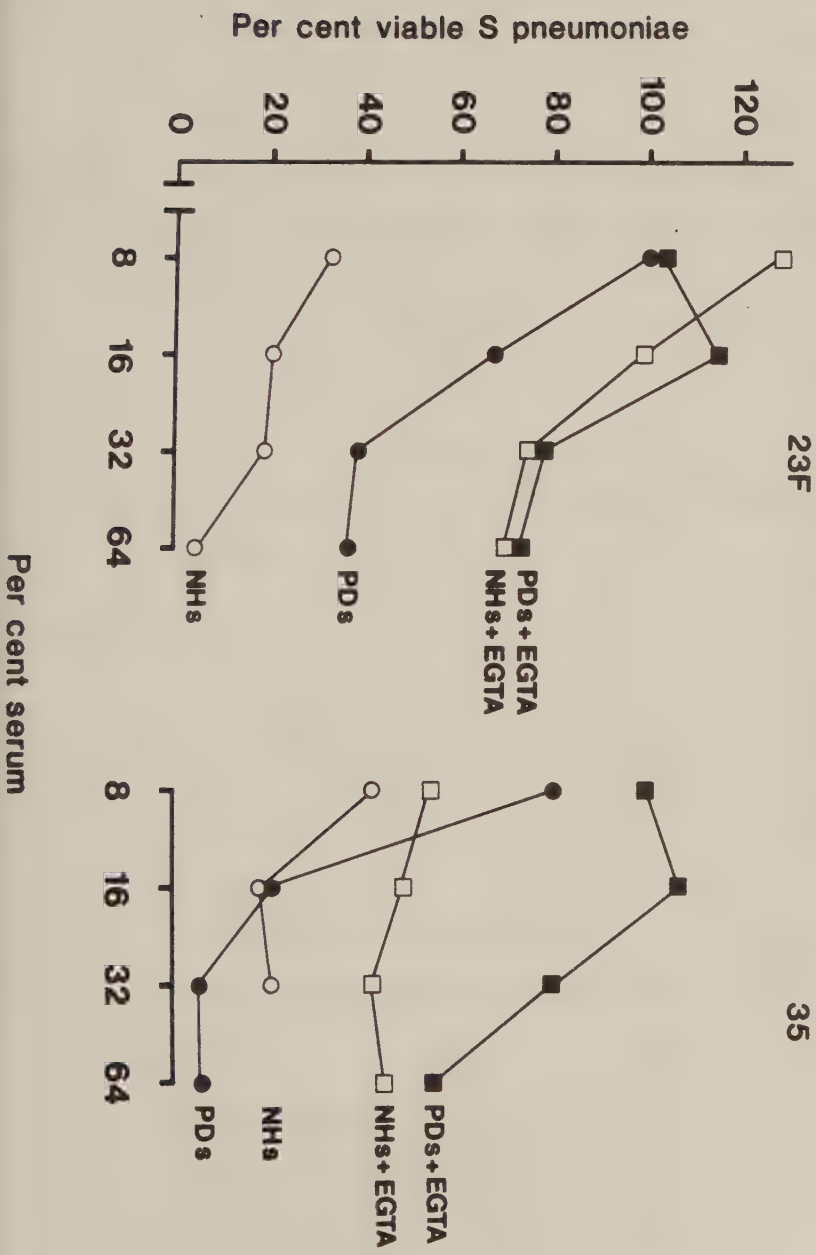


Figure 3

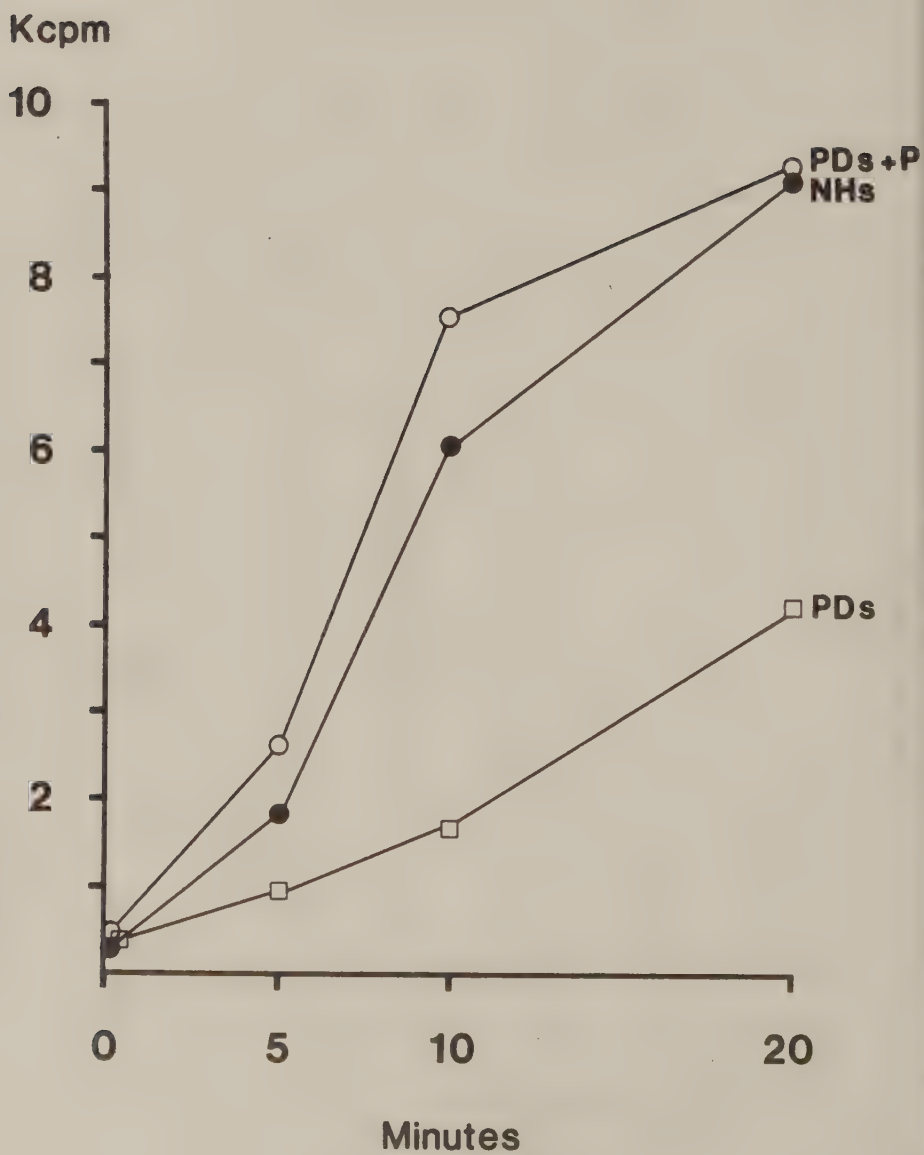


Figure 4

V

Opsonic and antibody responses to pneumococcal polysaccharide
types 6A, 19F and 23F after vaccination of immunocompromised
patients

Jean H. Braconier, Freddy Karup Pedersen, Håkan Odeberg
and Christer Rosén

Running head: Pneumococcal vaccination

From the Department of Infectious Diseases, Research Department 2,
E-bloket and Department of Otorhinolaryngology, University Hospital,
Lund, Sweden and the WHO Collaborating Centre for Reference and
Research on Pneumococci, Statens Seruminstitut, Copenhagen, Denmark

Summary

Opsonic and antibody responses to pneumococcal polysaccharide types 6A, 19F and 23F were evaluated before and after vaccination with a 14-valent pneumococcal vaccine in 25 patients splenectomized due to trauma, non-malignant or malignant disease and in 8 non-splenectomized patients with malignant disease. In approximately 50 % of the tests, a 2-fold or greater increase in antibody concentrations and a significantly enhanced opsonization of pneumococci was found.

A close correlation between antibody increase and enhancement of opsonization was demonstrated. Ninety-three % of paired samples with postimmunization antibody increase above 150 ELISA units showed significantly enhanced opsonization. Increased postvaccination opsonic activity and antibody levels were infrequently accompanied by increased granulocyte chemotactic activity of the serum.

No significant difference in antibody and opsonic response to vaccination was found between the groups of patients, except for patients with Hodgkin's disease receiving chemotherapy, who had a reduced immunization response. Prevacination antibody concentration, type of antigen or age of the patients did not influence the outcome of vaccination.

Introduction

Anti-pneumococcal antibodies promote phagocytosis (opsonization) and subsequent killing of pneumococci via formation of antigen-antibody complexes, which bind and activate the complement system (27).

Pneumococcal polysaccharide vaccine is immunogenic and affords protection against infection with vaccine type pneumococci in young healthy adults (3,25). The antibody response to pneumococcal vaccination in various immunocompromised patients, known to be at high-risk of pneumococcal infection, however has been found reduced (9,14,21,22,24). Furthermore, 25-40 % of patients with a significant antibody response after vaccination have not demonstrated enhancement of type specific opsonization (9,12). Conclusive studies of clinical protection by the vaccine in high-risk groups are not available (1,8). Evaluation of vaccine efficacy, therefore presently must be based upon studies of serological response, and on knowledge about the correlation between increased antibody concentration and enhanced opsonization.

The purpose of the present investigation was to study serum opsonic activity and antibody responses to three pneumococcal polysaccharide antigens of different immunogenicity (10), after vaccination with a 14-valent pneumococcal vaccine of different groups of patients with high-risk for pneumococcal infection.

Material and methods

Bacteria. S. pneumoniae serotypes 6A, 19F and 23F were obtained from Statens Seruminstitut, Copenhagen, Denmark. The pneumococci were maintained on blood agar and the strains were renewed monthly from stock cultures in foetal calf serum at -80°C . The bacteria were cultured for four hours in a brain heart infusion broth (Difco laboratories, Detroit, Mi, USA) supplemented with 50 mg per ml cysteine chlorid in an anaerobic environment (GasPak, Becton, Dickinson and Co, Cockeysville, Md, USA) and harvested in the logarithmic growth phase, washed and suspended in Hanks' balanced salt solution (HBSS)

buffered with 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), pH 7.4 as earlier described (6).

Patients. The following groups of patients were studied: 1. eight individuals splenectomized due to trauma (mean age 29.4 years), 2. nine patients splenectomized because of non-malignant diseases (leukopenia, thrombocytopenia, spherocytosis, myelofibrosis and pancreatitis) (mean age 49.5 years), 3. eight patients splenectomized due to malignant diseases of the hematopoietic system, five with Hodgkin's disease and three with hairy cell leukemia (mean age 52.6 years), and 4. eight non-splenectomized patients with malignant hematopoietic diseases, seven with Hodgkin's disease and one with non-Hodgkin lymphoma, were also studied (mean age 42.5 years).

Granulocytes. Granulocytes from healthy individuals were isolated from peripheral blood using Isopaque-Ficoll . After lysis of red cells with 0.87 % NH_4Cl , the granulocytes were washed and suspended in HEPES-HBSS as earlier described (20).

Sera. Sera were collected before and 1.4 - 2.5 months (mean for groups) after immunization with a 14-valent pneumococcal vaccine (Pneumovax, Merck Sharp & Dohme, West Point, PA, USA) containing polysaccharide types 1, 2, 3, 4, 6A, 7F, 8, 9N, 12F, 14, 18C, 19F, 23F and 25. The sera were stored in aliquote at -80°C .

Antibody determinations. Type specific anti-pneumococcal polysaccharide antibodies measured as total immunoglobulin were determined by enzyme-linked immunosorbent assay (ELISA) as earlier described (13).

Phagocytic killing of bacteria. Opsonization was measured as granulocyte phagocytic killing of pneumococci, since ingested S. pneumoniae are rapidly killed by granulocytes (6). Approximately 5×10^6 organisms were incubated for 30 minutes at 37°C with 10^6 granulocytes in serum diluted to 16 %, in a total volume of 250 μl .

Surviving microorganisms were measured by colony counting as previously described (6). If bacterial killing at 16 % serum was less than 20 % or greater than 80 % in both pre- and postvaccination samples, the serum pair was retested at 32 and 8 % concentrations, respectively. The variation of the assay system was evaluated at different levels of bacterial killing. The SD at 20 % bacterial killing was ± 4.6 % and at 80 % killing ± 6.3 %. Increased opsonic activity in postvaccination sera was defined as an increase in bacterial killing exceeding 2 SD.

Some patient sera were heat inactivated at 56°C for 30 minutes and tested with serum from a patient with selective and complete deficiency of IgG subclass 2 (IgG2) as a complement source (IgG subclass determination kindly performed by Dr. V-A. Oxelius, Lund, Sweden). The IgG2 deficient serum had normal levels of C3, C4, C5, factor B and properdin (kindly determined by Dr. A. Sjöholm, Lund, Sweden) and was free of antibodies to pneumococcal polysaccharides types 6A, 19F and 23F measured with ELISA.

Granulocyte chemotaxis. The chemotaxis of granulocytes from healthy individuals was studied on agarose plates with a method modified from Nelson et al (1975) (19). Approximately 10^9 S. pneumoniae serotypes 6A, 19F and 23F were incubated in 12.5 % of pre- or postvaccination serum for 4 minutes at 37°C. A serum concentration of 12.5 % had been found suitable, since it supported a substantial but suboptimum chemotaxis. After heat inactivation (56°C, 30 minutes) followed by centrifugation, the supernatants were used as chemoattractants. 1×10^6 granulocytes migrated under agar towards wells containing 10 μ l of chemoattractant. Incubations were for three hours at 37°C in a humidified atmosphere. The under-agarose technique measures simultaneously the random migration towards wells containing control medium (distance B) and the distance covered by the cells in response to a chemoattractant (distance A). The chemo-

tactic index was expressed by the ratio A/B. The SD of the method for experiments performed simultaneously was 5 %.

Statistical calculations. Comparison of the numbers of patients responding in each group and to each serotype was done by means of χ^2 -test or Fisher's exact test.

Results

The serum opsonic activity and the arbitrary concentration in ELISA units of antibodies against S. pneumoniae serotypes 6A, 19F and 23F in the four groups of patients before and after vaccination are shown in figures 1-4. Pneumococcal vaccination induced increased opsonization of serotypes 6A and 23F in 47 % each and of serotype 19F in 63 % of the patients ($p > 0.05$). The antibody concentrations rose more than 2-fold against serotypes 6A, 19F and 23F in 58 %, 53 % and 56 %, respectively, of the patients. A close correlation was found between antibody fold increase and enhancement of opsonization. Serum pairs with antibody concentration increases of more than 150 arbitrary units showed significantly enhanced opsonization in 93 % (43/46) of the assays, while only 13 % (6/48) with antibody concentration increase less than 100 units were associated with increased opsonization. In sera with at least 2-fold antibody rise after vaccination, 83 % (45/54) of the assays showed increased opsonic activity.

Five serum pairs with discordance between opsonic and antibody response to vaccination, were also tested for opsonic activity after heat-inactivation and with replacement of complement by addition of non-immune serum. Identical results with those of the non-inactivated sera were found.

No difference in the response to immunization among patients with low (≤ 50 units) or high (≥ 200 units) prevaccination antibody concentrations was found. In serum pairs with low prevaccination antibody levels, increased opsonic activity and 2-fold or greater antibody rise were found in 56 % and 59 % respectively, of the assays. In serum pairs with high prevaccination levels, the corresponding figures were 59 % and 50 % respectively. High prevaccination antibody levels were found more often against serotype 19F than against 6A and 23F ($p < 0.05$).

The underlying disease had little influence on the outcome of vaccination. Postvaccination sera showed in the different groups of patients, increased opsonic activity in 46 % to 63 % of the assays and at least 2-fold antibody rise in 50 % to 63 % (Figures 1-4). These differences between the various groups of patients were not significant. In seven patients splenectomized less than a year before vaccination, increased opsonization was found in 76 % (16/21) of the assays. In 18 patients vaccinated more than a year after the splenectomy, 48 % (26/54) showed increased opsonization ($p < 0.05$). A 2-fold antibody concentration increase was found in 81 % of the assays with serum pairs from patients with less than a year's interval between vaccination and splenectomy and in 46 % of assays with serum from patients with more than a year's interval ($p < 0.01$).

Advanced age was well compatible with a favourable outcome of vaccination. In 24 assays from eight patients more than 60 years old, increased opsonization was found in 67 % and a more than 2-fold antibody increase in 52 %.

Five patients with Hodgkin's disease were vaccinated and studied during chemotherapy, and two begun chemotherapy soon after immunization, before the postvaccination sample was drawn. Among these patients

increased opsonization was found in 24 % (5/21) of the assays and, a 2-fold or greater antibody increase in 33 %, which is significantly lower than in the remainder of our patients ($p < 0.01$ and $p < 0.05$, respectively). Two other patients with Hodgkin's disease, vaccinated during radiotherapy responded with increased opsonic activity and 2-fold antibody increase in 6/6 experiments.

Chemotactic activity was studied in serum pairs from five patients with increased opsonic activity and at least 2-fold increase of antibody concentration after vaccination. No increase of granulocyte chemotaxis was induced by S. pneumoniae serotype 6A and post-vaccination sera. In experiments with serotypes 19F and 23F increased chemotaxis was found in two and one postvaccination sera, respectively.

Discussion

Phagocytosis is essential for the human defence against S. pneumoniae (27). The capsule of the pneumococcus has antiphagocytic properties (17) and effective opsonization of this organism require activation of the complement factor C3 by specific anti-capsular antibodies (7).

In the present study, it was found that approximately 50 % of patients with high-risk of pneumococcal infection, after immunization showed at least 2-fold antibody increase and enhanced opsonic activity against the tested pneumococcal serotypes. A close correlation between antibody increase and enhancement of opsonization was found, provided that increase in antibody concentration was above 150 ELISA units. A similar correlation was not found for antibody increase below that value, which only may be a reflexion of the sensitivity of the opsonic assay. In a recent study it was estimated that the mean, minimal pneu-

mococcal antibody concentration conferring immunity against six pneumococcal serotypes was about 130 ELISA units (16). This is about the same concentration as we found required for demonstration of improved opsonization.

Giebink et al (9) studied the opsonic activity after vaccination against serotypes 3, 6A and 7F in children splenectomized due to traumatic splenic laceration. Forty-two % of the children with at least 2-fold antibody increase after vaccination failed to show an autologous opsonic response. Simberkoff et al (24) found after vaccination increased opsonic activity against serotypes 1 and 3, in only 50 % and 36 % of adult chronic hemodialysis patients, respectively. A poor correlation between postvaccination antibody levels and opsonic activity was found for serotype 1. Also Johansen and Karup Pedersen (12) in a study of splenectomized individuals and healthy adults found, that 30 % of patients with a significant response to vaccination did not display any increase in type specific opsonization.

Much of the discrepancies in opsonic activity between these and our investigations are likely to be methodological. The outcome of the phagocytic assay used in our study to evaluate the opsonic activity of serum is dependent on the concentration of the serum employed (6). In the present investigation the opsonic activity has been evaluated with serum concentrations which supported a moderately efficient phagocytic killing (20-80 %) of the pneumococci. Simberkoff et al (24) imposed a high request on increase in opsonic activity after immunization and the serotypes used in both their and in the Danish (12) study, particularly serotype 3, is provided with an unusually large antiphagocytic capsule (4). The low number of increased opsonic assays after vaccination, found by Giebink et al (9) is probably due to the use of only one serum concentration.

The similar immunogenicity of type antigens 6A, 19F and 23F,

contrasting to other studies (10, 15), could be due to the large individual variation in response to polysaccharide antigens (15), rendering conclusions less reliable with the small groups studied. This might also explain the discrepancy between our finding of a more favourable outcome of vaccination when performed less than a year after vaccination and the observations by Giebink et al (9). They found no difference in response to pneumococcal vaccination between ten patients immunized within four weeks of splenectomy and five children vaccinated about five years after splenectomy. Advanced age did not influence the result of pneumococcal vaccination. Similar observations have been made by Hilleman et al (10).

The impaired antibody response after pneumococcal vaccination of patients with Hodgkin's disease treated with radiation and chemotherapy is in agreement with other studies (2, 18, 22). It has therefore been recommended that pneumococcal vaccination is performed either before or some time after treatment for Hodgkin's disease (23).

The main role of antibody in opsonization of S. pneumoniae is probably to mediate the fixation of opsonic complement, C3b, to the bacterial surface (7). During the further activation of the complement system, C5a, the major chemoattractant of serum is formed (26). In the present study five serum pairs with increased opsonic activity and antibody concentrations were evaluated for granulocyte chemotactic activity. The results indicate that increased levels of opsonic antibodies after pneumococcal vaccination are not regularly accompanied by increased granulocyte chemotaxis.

Antibodies in this study are measured as total immunoglobulin, including non-opsonizing IgA antibodies. Our results, which indicate a good correlation between opsonic activity and ELISA-measured antibodies, thus suggest that the major part of the antibodies found after vaccination are of IgG and of IgM class, as suggested by others (5, 11,

15). The correlation between anti-pneumococcal antibody and opsonic activity makes judgement of vaccine efficacy (short of clinical trials) based upon the simpler antibody determinations possible.

Acknowledgements

The technical assistance of Mrs. Annie Kleis Nielsen and Mrs. Britt Turesson is much appreciated.

This investigation was supported by the Swedish Medical Research Council, the Medical Faculty of Lund and Malmöhus läns landsting.

Legends to figures

Figure 1. Antibody concentration and opsonic response to S. pneumoniae serotypes 6A, 19F and 23F in patients splenectomized due to trauma, before (B) and after (A) immunization with a 14-valent pneumococcal vaccine. Antibody concentration, measured with ELISA, is expressed in arbitrary units. Significant increase of pneumococcal opsonization after immunization is indicated by closed symbols and open symbols denote unchanged opsonic activity.

Figure 2. Patients splenectomized due to non-malignant diseases. Symbols are identical with those of figure 1.

Figure 3. Patients splenectomized due to malignant diseases. Symbols are identical with those of figure 1.

Figure 4. Non-splenectomized patients with malignant diseases. Symbols are identical with those of figure 1.

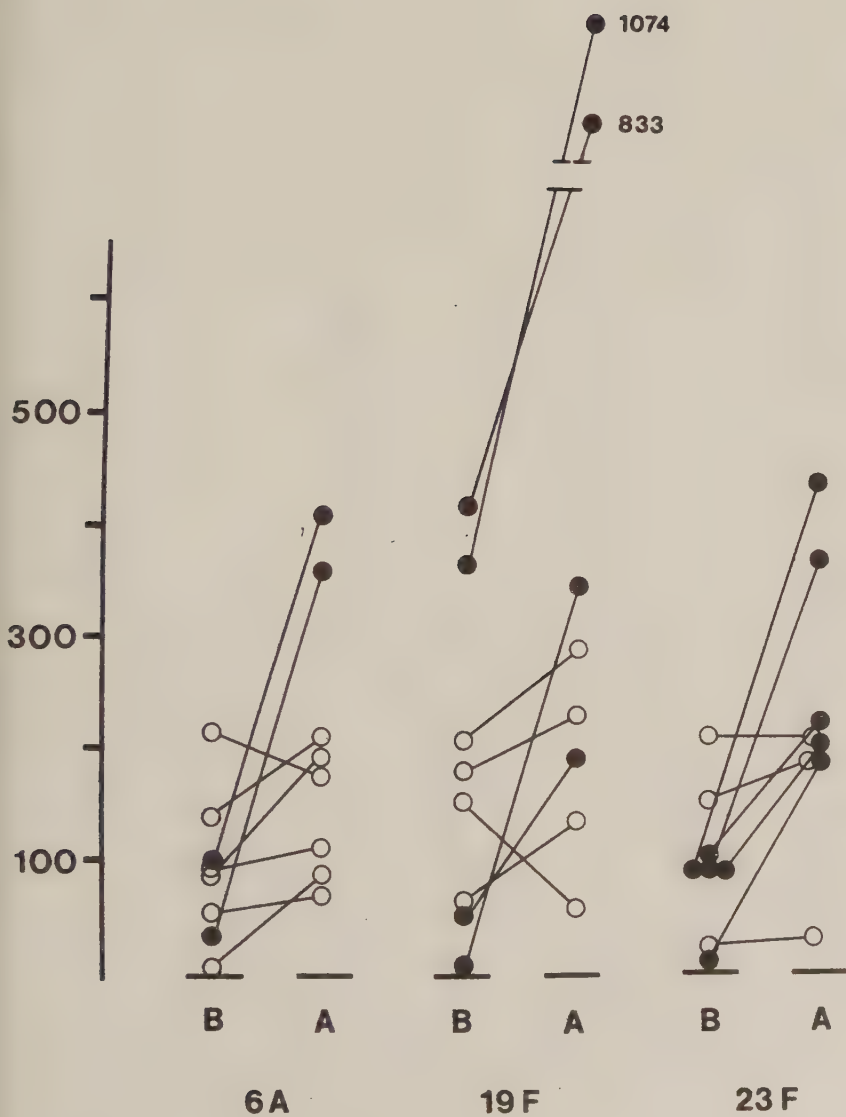


Figure 1

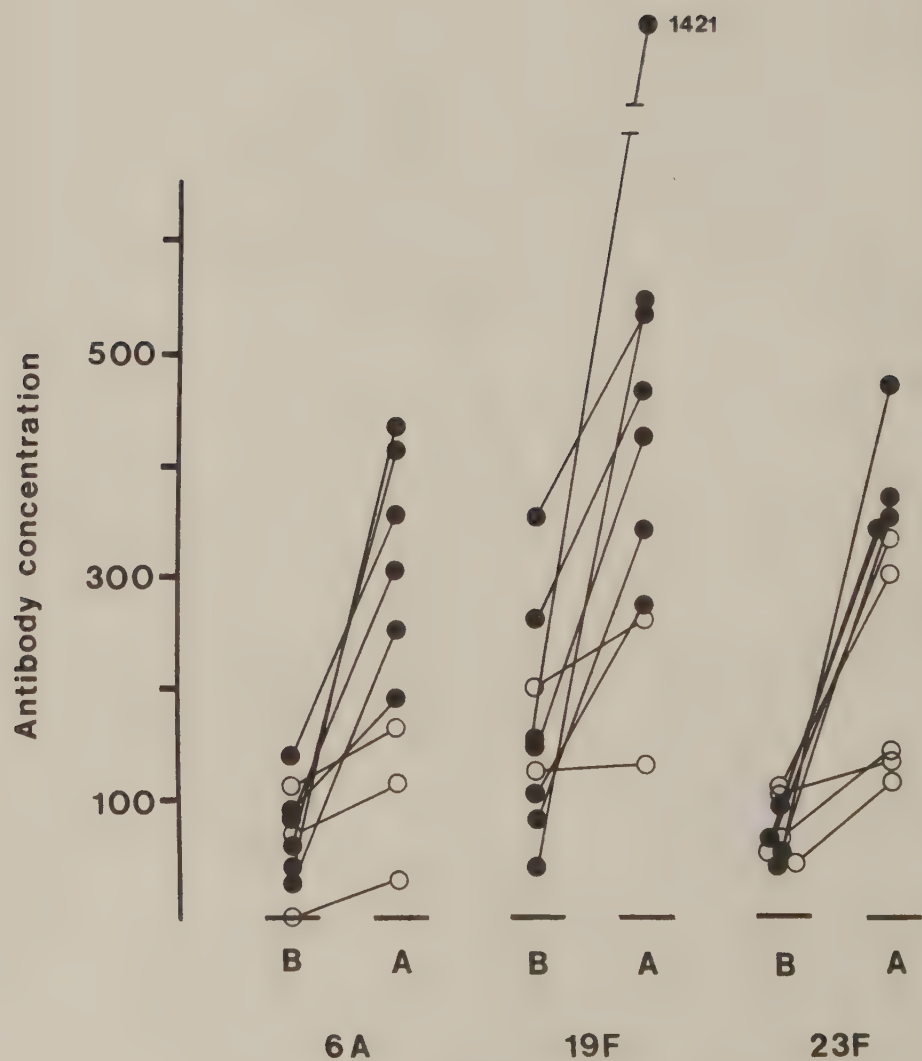


Figure 2

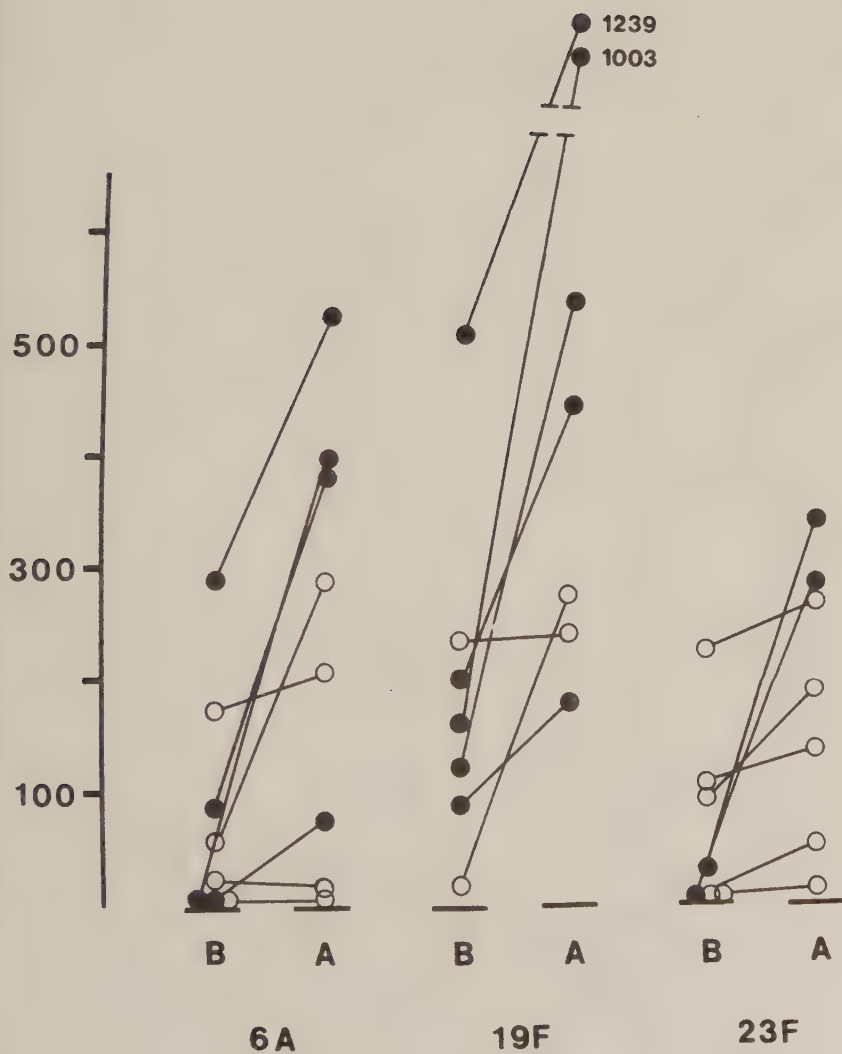


Figure 3

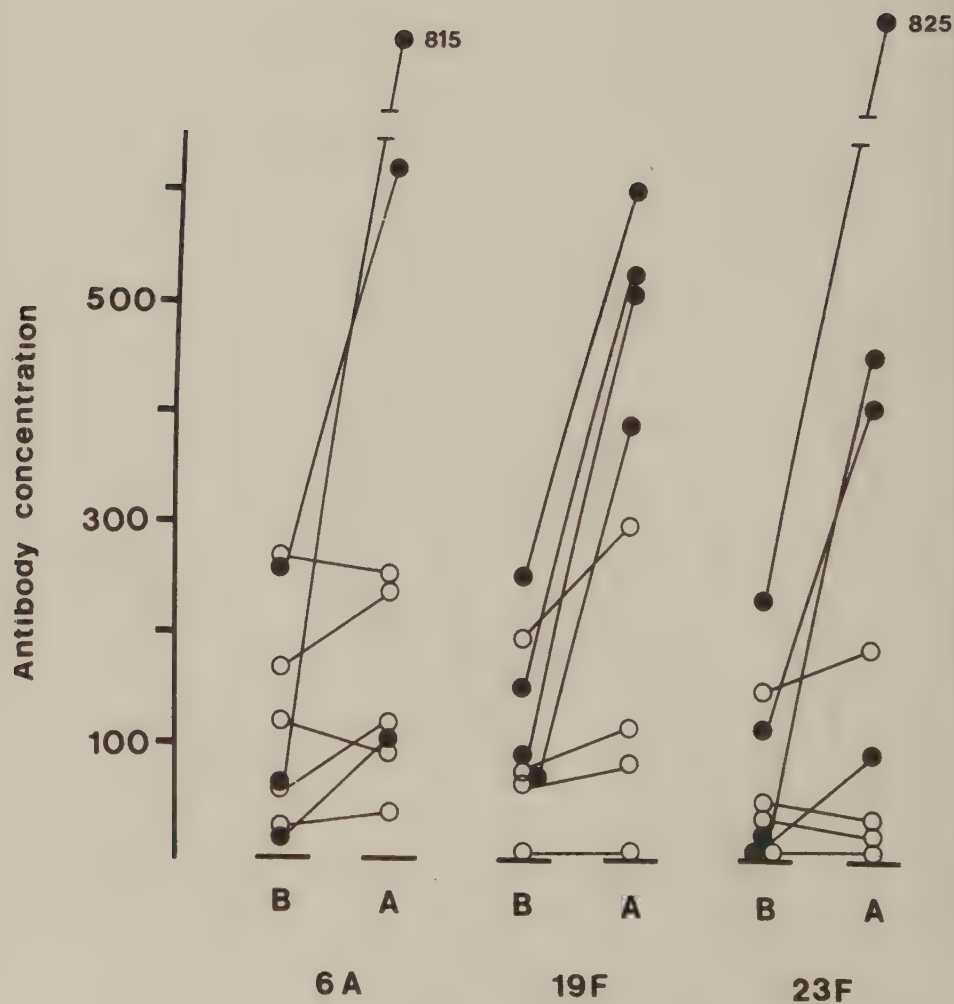


Figure 4

References

1. Amman, A.J., Addiego, J., Wara, D.W., Lubin, B., Smith, W.B. & Mentzer, W.C.: Polyvalent pneumococcal-polysaccharide immunization of patients with sickle-cell anemia and patients with splenectomy. *N Engl J Med* 297:897, 1977.
2. Amman, A.J., Schiffman, G., Addiego, J.E., Wara, W.M. & Wara, D.W.: Immunization of immunocompromized patients with pneumococcal polysaccharide vaccine. *Rev Infect Dis* 3:S160, 1981.
3. Austrian, R., Douglas, R.M., Schiffman, G., Coetsee, A.M., Koornhof, H.J., Hayden-Smith, S. & Reid, R.D.W.: Prevention of pneumococcal pneumonia by vaccination. *Trans Assoc Am Phys* 89:184, 1976.
4. Austrian, R.: Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. *Rev Infect Dis* 3:S1, 1981.
5. Barret, D.J., Amman, A.J., Stermark, S. & Wara, D.W.: Immunoglobulin G and M antibodies to pneumococcal polysaccharides detected by enzyme-linked immunosorbent assay. *Infect Immun* 27:411, 1980.
6. Braconier, J.H. & Odeberg, H.: Granulocyte phagocytosis and killing of virulent and avirulent serotypes of Streptococcus pneumoniae. *J Lab Clin Med* 100:279, 1982.
7. Brown, E.J., Hosea, S.W., Hammer, C.H., Burch, C.G. & Frank, M.M.: A quantitative analysis of the interactions of antipneumococcal antibody and complement in experimental pneumococcal bacteremia. *J Clin Invest* 69:85, 1982.
8. Broome, C.V.: Efficacy of pneumococcal polysaccharide vaccines. *Rev Infect Dis* 3:S82, 1981.
9. Giebink, G.S., Foker, J.E., Kim, Y. & Schiffman, G.: Serum antibody and opsonic responses to vaccination with pneumococcal

- capsular polysaccharide in normal and splenectomized children. *J Infect Dis* 141:404, 1980.
10. Hilleman, M.R., Carlson, A.J. Jr., McLean, A.A., Vella, P.P., Weibel, R.E. & Woodhour, A.F.: Streptococcus pneumoniae polysaccharide vaccine: Age and dose responses, safety, persistence of antibody, revaccination and simultaneous administration of pneumococcal and influenza vaccines. *Rev Infect Dis* 3:S31, 1981.
 11. Hosea, S.W., Brown, E.J., Burch, C.G., Berg, R.A. & Frank, M.M.: Impaired immune response of splenectomized patients to polyvalent pneumococcal vaccine. *Lancet* 1:803, 1981.
 12. Johansen, K.S. & Karup Pedersen, F.: Antibody response and opsonization after pneumococcal vaccination in splenectomized children and healthy persons. *Acta Pathol Microbiol Immunol Scand. Sect C* 90:205, 1982.
 13. Karup Pedersen, F. & Henrichsen, J.: Detection of antibodies to pneumococcal capsular polysaccharides by enzyme-linked immunosorbent assay. *J Clin Microbiol* 15:372, 1982.
 14. Karup Pedersen, F., Henrichsen, J. & Schiffman, G.: Antibody response to vaccination with pneumococcal capsular polysaccharides in splenectomized children. *Acta Paediatr Scand* 71:451, 1982.
 15. Karup Pedersen, F.: Antibody response to pneumococcal vaccine in splenectomized children. *Acta Pathol Microbiol. Sect C*. In press.
 16. Karup Pedersen, F., Henrichsen, J. & Schiffman, G.: Comparison of enzyme-linked immunosorbent assay and radioimmunoassay for detection of anti-pneumococcal polysaccharide antibodies. To be published.
 17. McCarty, M.: Pneumococci. In: *Microbiology*, p. 694 (ed. B.D. Davis, R. Dulbecco, H.N. Eisen, H.S. Ginsberg, W.B. Wood & M. McCarty). Harper & Row, Publishers, New York 1973.
 18. Minor, D.R., Schiffman, G. & McIntosh, L.S.: Response of patients with Hodgkin's disease to pneumococcal vaccine. *Ann Intern Med* 90:

887, 1979.

19. Nelson, R.D., Quie, P.G. & Simmons, R.L.: Chemotaxis under agarose: a new and simple method for measuring chemotaxis and spontaneous migration of human polymorphonuclear leukocytes and monocytes. *J Immunol* 115:1650, 1975.
20. Odeberg, H., Olofsson, I. & Olsson, I.: Granulocyte function in chronic granulocytic leukemia. I. Bactericidal and metabolic capabilities during phagocytosis in isolated granulocytes. *Br J Haematol* 29:427, 1975.
21. Schmid, G.P., Smith, R.P., Baltch, A.L., Hall, C.A. & Schiffman, G.: Antibody response to pneumococcal vaccine in patients with multiple myeloma. *J Infect Dis* 143:590, 1981.
22. Siber, G.R., Weitzman, S.A., Aisenberg, A.C., Weinstein, H.J. & Schiffman, G.: Impaired antibody response to pneumococcal vaccine after treatment for Hodgkin's disease. *N Engl J Med* 299:442, 1978.
23. Siber, G.R., Weitzman, S.A. & Aisenberg, A.C.: Antibody response of patients with Hodgkin's disease to protein and polysaccharide antigens. *Rev Infect Dis* 3:5144, 1981.
24. Simberkoff, M.S., Schiffman, G., Katz, L.A., Spicehandler, J.R., Moldover, N.H. & Rahal, J.J. Jr.: Pneumococcal capsular polysaccharide vaccination in adult chronic hemodialysis patients. *J Lab Clin Med* 96:363, 1980.
25. Smit, P., Oberholzer, D., Hayden-Smith, S., Koornhof, H.J. & Hilleman, M.R.: Protective efficacy of pneumococcal polysaccharide vaccines. *JAMA* 238:2613, 1977.
26. Wilkinson, P.C.: Leukocyte locomotion and chemotaxis: Effects on bacteria and viruses. *Rev Infect Dis* 2:293, 1980.
27. Winkelstein, J.A.: Opsonins: their function, identity and clinical significance. *J Pediatr* 82:747, 1973.

Pneumococcal vaccination. Opsonic and antibody response to clinically important serotypes, not included in the vaccine.

Jean Henrik Braconier, Erling Brandsberg Myhre and Håkan Odeberg

Department of Infectious Diseases and Research Department 2,
E-blocket, University Hospital, S-221 85 Lund, Sweden.

Running head: Pneumococcal vaccination.

This work was supported by the Swedish Medical Research Council (B82-17X-05430-04), the Medical Faculty of Lund and the foundation of Österlund. The technical assistance of Mrs Britt Thuresson is greatly appreciated.

Informed consent was obtained from the patients and the guidelines of the Ethical Committee of University of Lund were followed.

Please address requests for reprints to Dr. Jean Henrik Braconier,
Department of Infectious Diseases, University Hospital, S-221 85
Lund, Sweden.

Abstract

Serum antibodies and opsonic activity to pneumococcal serotypes 6B, 9V and 19A were measured in 10 patients before and after immunization with a dodecavalent pneumococcal vaccine. The capsular polysaccharides of these serotypes are not included among, but are antigenically related to the vaccine polysaccharides. Patients responding to immunization with at least a twofold increase in serum antibodies to vaccine polysaccharides 6A, 19F and 23F were studied. Antibodies to serotypes 6B, 9V and 19A were measured by a staphylococcal protein-A binding assay. A twofold or greater increase in antibodies to serotypes 6B, 9V and 19A was found in postvaccination serum from 10, 3 and 7 patients, respectively. Increased opsonic activity towards these serotypes was demonstrated in 8, 4 and 7 patients, respectively. These results indicate that humans responding to pneumococcal vaccination, may also develop opsonic antibodies to other clinically important pneumococcal serotypes. The degree of cross-immunization appears to vary between individuals and between different pneumococcal serotypes.

Introduction

The polysaccharide capsule is the major antigenic component of the pneumococcal cell (1). There are 83 serologically defined capsular polysaccharide types, many of which are crossreactive (2). Antigenically related polysaccharide types form pneumococcal groups indicated in the Danish system of nomenclature by a common number; related types within the groups are distinguished from one another by letter designation (2).

Purified capsular polysaccharides are immunogenic in man, inducing a type-specific antibody response (3). A currently employed pneumococcal vaccine contains 14 different polysaccharides corresponding to the pneumococcal serotypes most frequently producing infectious diseases (3, 4). Other serotypes, however, may also be of clinical importance. Over 14 % of the serious pneumococcal infections in the USA in 1978-1979 were caused by serotypes 6B, 9V and 19A, representing capsular polysaccharides not included in the vaccine (5). The vaccine may nevertheless provide protection against infection by these serotypes as well, since it contains antigenically related capsular polysaccharides 6A, 9N and 19F. The extent of cross-immunogenicity, and the possible protection may vary between the serotypes (6, 7, 8). The importance of serotypes 6B and 19A is further underlined by observation of multiple antibiotic resistance in some strains of these serotypes (9, 10).

In the present study we have determined the effect of vaccination on serum antibody levels and opsonic activity towards pneumococcal serotypes 6B, 9V and 19A. The immunization was found to induce increased opsonic antibody activity to serotypes 6B and 19A in most patients, while only a few patients showed enhanced opsonization of serotype 9V.

Material and methods

Bacteria

Streptococcus pneumoniae serotypes 6A, 6B, 9N, 9V, 19A, 19F, 31, 36 and 38 were obtained from Statens Seruminstitut, Copenhagen, Denmark. The pneumococci were cultured anaerobically on blood agar and were renewed monthly from stock cultures stored in foetal calf serum at -80°C as previously described (11). The bacteria were grown for four hours in brain heart infusion broth (Difco laboratories, Detroit, Mi., USA) washed twice and suspended in a modified HEPES- Hanks' balanced salt solution (HBSS) buffer (11) to a final concentration of approximately 5×10^7 viable organisms per ml.

Staphylococcus aureus strain 502A was grown in Bacto antibiotic medium 3 (Difco) and suspended as described earlier (12).

Sera. Pre- and postvaccination (the mean interval between samples was six weeks) sera had been collected during an earlier vaccine-study in which splenectomized patients and other risk groups had been immunized with a 14-valent pneumococcal vaccine (Pneumovax, Merck Sharp & Dohme, West Point, Pa., USA).¹ Serum pairs demonstrating a twofold or greater increase in antibodies to capsular polysaccharide types 6A, 19F and 23F measured by ELISA were selected for the present study. The mean fold antibody increase was 7.5, 5.2 and 4.4 respectively.

Granulocytes. Granulocytes from healthy individuals were isolated from peripheral blood using Isopaque-Ficoll as earlier described (13).

Radiolabeling of protein A. Purified staphylococcal protein A (Pharmacia Fine Chemicals, Uppsala, Sweden) was labeled by ^{125}I with a modified chloramine-I method (14). Free, unreacted isotope was removed by gel filtration on a prepacked Sephadex G-25 column (PD-10,

Pharmacia Fine Chemicals) eluted with phosphate buffered saline (PBS, 0.12 M NaCl, 0.03 M phosphate, pH 7.2) containing 0.02 % sodium azide and 0.1 % bovine serum albumin (BSA). The specific activity of the preparation was 0.4 mCi per mg protein A.

Quantitation of pneumococcal antibodies. Specific antibodies were measured by a protein-A binding technique (15, 16). S. pneumoniae harvested in log-phase (approximately 5×10^6 organisms) were suspended in HEPES-HBSS buffer and mixed in polystyrene tubes with 40 μ l serum in a total volume of 250 μ l. The tubes were incubated under slow agitation for 15 minutes at 37°C. The bacteria were washed twice and resuspended in 200 μ l buffer containing 0.1 % BSA and mixed with 20 μ l 125 I-protein-A (approximately 2 μ g, 70000 cpm). After 30 minutes at room temperature, 4 ml HEPES-HBSS-BSA buffer was added and the bacteria were sedimented by centrifugation at 1000 x G. The bacterial pellet was washed once and the radioactivity in the bacterial pellet was measured (80000 Gamma Sample Counter, LKB, Stockholm, Sweden). The amount of serum antibodies bound to the bacterial surface was expressed as the difference in 125 I-protein-A binding to bacteria preincubated in the presence or absence of serum. Binding of protein A to pneumococci was linearly dependent on the amount of serum present during the preincubation and was in the absence of serum less than 0.2 % of the added radioactivity. Measurement by the protein-A binding assay of the relative antibody titres to serotypes 6A and 19F in four pre- and postvaccination sera yielded values closely similar to those previously obtained by ELISA.

Pre- and postvaccination sera were tested in parallel and in duplicates. The results were corrected for non-specific binding and for the natural decay of the isotope. The standard error of the mean was less than ± 25 % of the mean for the duplicate samples. The antibody fold increase was calculated as the quotient between post- and

prevaccination values.

Phagocytic killing of bacteria. Opsonization was measured as granulocyte phagocytic killing of pneumococci, since ingested S. pneumoniae are rapidly killed by granulocytes (11). Approximately 5×10^6 organisms were incubated for 30 minutes at 37°C with 10^6 granulocytes in serum diluted to 16 % with HEPES-HBSS buffer in a total volume of 250 μ l. Surviving microorganisms were measured by colony counting as previously described (11). If bacterial killing at 16 % serum was less than 20 % or greater than 80 % in both pre- and postvaccination samples, the serum pair was retested at 32 and 8 % concentrations, respectively. The variation of the assay system was evaluated at different levels of bacterial killing. The SD at 20 % bacterial killing was ± 4.6 % and at 80 % killing ± 6.3 %. Increased opsonic activity in postvaccination sera was defined as an increase in bacterial killing exceeding 2 SD.

Some patient sera were heat inactivated at 56°C for 30 minutes and tested with serum from a patient with selective and complete deficiency of IgG subclass 2 (IgG2) as a complement source (IgG subclass determination kindly performed by Dr. V-A. Oxelius, Lund, Sweden). The IgG2 deficient serum had normal levels of C3, C4, C5, factor B and properdin (kindly determined by Dr. A. Sjöholm, Lund, Sweden) and was free of antibodies to pneumococcal polysaccharides types 6A, 19F and 23F measured with ELISA by Dr. J. Henrichsen, Statens seruminstitut, Copenhagen, Denmark.

Results

Serum antibodies to *S. pneumoniae* serotype 6B, 9V and 19A. Ten pairs of human pre- and postvaccination sera with at least a twofold increase in ELISA-measured antibodies to the vaccine polysaccharide types 6A, 19F and 23F were tested with a protein-A binding assay for antibodies to pneumococcal serotypes 6B, 9V and 19A (Fig 1).

All ten postvaccination sera contained at least twofold higher concentrations of antibodies to serotype 6B organisms. The geometric mean fold antibody increase was 5.0.

Eight of the 10 postvaccination sera showed a twofold or greater antibody increase to serotype 19A organisms. The mean fold antibody rise was 3.7, although the extent of increase varied greatly.

In contrast only three postvaccination sera showed at least twofold antibody increase to serotype 9V. Antibody levels in the remaining seven sera were either unchanged or only slightly elevated. The mean fold antibody increase was 1.9.

To exclude the possibility that the weak antibody rise to serotype 9V was due to selection of sera with inferior response to vaccine polysaccharide 9N, pre- and postvaccination antibody levels to serotype 9N organisms were determined. Four sera with low rise in antibody levels to serotype 9V (geometric mean fold increase 1.4) were tested with serotype 9N. All four sera showed a twofold or greater rise in antibodies to serotype 9N, with a mean fold increase of 3.0.

Pre- and postvaccination sera from one patient with a substantial postimmunization antibody rise to serotypes 6B, 9V and 19A were examined for antibodies to *S. pneumoniae* serotype 31, 36 and 38. Serotype 31 is antigenically unrelated to the vaccine polysaccharides and was included as a test organism for antibodies to antigen on the cell

surface other than the pneumococcal capsule. Serotype 36 has as antigenic determinant 9e in common with the vaccine polysaccharide 9N (2), and the vaccine type 25 and serotype 38 both contain the antigen 25b. A slight increase in antibodies to all three serotypes was found after vaccination. However, the antibody rise was less than 25 % of the pre-vaccination level. These results indicate that the antibody rise to serotypes 6B, 9V and 19A were not directed towards common pneumococcal antigens.

Opsonization of bacteria. The opsonic activity of pre- and post-vaccination sera is shown in figure 1. Eight sera showed increased opsonic activity towards serotype 6B after immunization. Two other post-vaccination sera displayed no increase in opsonic activity, although they possessed increased antibody levels. Addition of non-immune serum (IgG2-deficient), as a source of complement, to one of these serum pairs (previously heat-inactivated) did not increase opsonic activity in the post-vaccination sample.

The three post-vaccination sera with a substantial increase of antibodies to serotype 9V produced greater opsonization of these organisms. Enhanced opsonization was also observed in one postvaccination serum sample with low rise of antibodies. This serum was re-tested after heat-inactivation with non-immune serum as a complement source, and the increased opsonic capacity to serotype 9V was confirmed. The other six sera with little or no antibody rise after immunization showed unchanged opsonic capacity.

Seven post-vaccination sera displayed increased opsonic activity to serotype 19A. The increase was also apparent in two of these sera, which were tested after heat-inactivation and repletion of complement by addition of non-immune serum. Three sera with a weak antibody rise to serotype 19A showed no change of opsonic capacity.

Three serum pairs with more efficient opsonization of serotypes

6B, 9V and 19A after immunization were tested for opsonic capacity to serotypes 31, 36 and 38. No increase in phagocytic killing was found after vaccination. Opsonization of S. aureus 502A was also unchanged in two of these serum pairs.

Discussion

In the present investigation we explored the capacity of a widely used dodecavalent pneumococcal polysaccharide vaccine to induce increased antibody and opsonic activity to pneumococcal serotypes not included, but antigenically related to the polysaccharides of the vaccine.

The study was performed with sera from patients who had responded to immunization by high levels of serum antibodies to the vaccine polysaccharide types 6A, 19F and 23F. It was found that the patients to a variable degree also developed antibodies and increased opsonic activity to the clinically important (5) pneumococcal serotypes 6B, 9V and 19A, whose capsular polysaccharides are not present in the vaccine. Appreciable increase of antibody concentration and of opsonic activity to serotypes 6B and 19A was found in a majority of the studied sera. However, only a few patients developed increased levels of opsonic antibodies to serotype 9V.

The biological activity of the antibodies was studied by the effect of serum on killing of S. pneumoniae by granulocytes. Killing of encapsulated pneumococci by granulocytes requires phagocytosis, which is dependent upon opsonization by serum (17). A twofold or greater increase of anti-pneumococcal antibodies following immunization was usually associated with enhanced opsonization of serotypes 6B, 9V and 19A. Unchanged opsonic activity was generally observed in

sera from patients with a weak antibody response. We found no difference in opsonic activity between heat-inactivated patient sera, supplemented with an external complement source and the fresh patient sera. These results indicate that the enhanced opsonic capacity in the post-vaccination sera was due to increased amounts of anti-pneumococcal antibodies.

Serological cross-reactivity between different pneumococcal serotypes has been investigated in the past, both in immunized rabbits and in small groups of vaccinated healthy humans. Demonstration of elevated antibody titres to serotype 6B in all our postvaccination sera confirms earlier reports suggesting extensive cross-reactivity with 6A polysaccharide (6). In fact, no difference in antibody levels to polysaccharide types 6A and 6B was seen, when healthy adults were immunized with a vaccine containing either 6A, or 6A and 6B polysaccharides (6). The weak cross-immunogenicity between 9N and 9V polysaccharides suggested by our study is in agreement with the findings of Szu et al (7, 18).

Seven of the 10 patients studied developed high levels of antibodies cross-reacting with serotype 19A organisms. These results are different from the findings of Lee et al (8), who reported that 80 % of healthy volunteers immunized with 19F polysaccharide developed a greater than twofold antibody rise to type 19F. Only 20 % of the individuals showed a similar rise to type 19A polysaccharide. This discrepancy may be explained by the inclusion of patients with high antibody levels in our study. Thus, the geometric mean fold increase to serotype 19F among our patients was 5.2 compared to 2.8 in the report of Lee et al (8).

Pneumococcal antibodies were measured by a protein-A binding assay utilizing whole pneumococcal organisms as antigen. Antibodies bound to antigens exposed on the bacterial cell surface were detected

with purified radiolabeled protein A acting as an anti-immunoglobulin reagent with specificity for IgG1, IgG2 and IgG4 as well as for some IgA and IgM antibodies (19, 20). By carrying out experiments with an excess of antigen and protein A, a linear correlation between the amount of antibodies in the sample and the protein-A binding capacity was found. By this method, antibodies interacting with the polysaccharide capsule can not be distinguished from antibodies to other surface antigens. However, there are evidence suggesting that the antibodies measured in the present study were mainly directed towards the polysaccharide component. In individual sera, rising antibody levels were demonstrated towards serotypes 6B and 19A organisms, but not towards the unrelated serotype 9V, 31, 36 and 38, suggesting a type-specific antibody response, i.e. formation of anticapsular antibodies.

The virulence of encapsulated S. pneumoniae is attributed to the antiphagocytic properties of the polysaccharide capsule. The importance of specific anticapsular antibodies in the opsonization and phagocytosis of the pneumococci forms the basis for vaccination with purified polysaccharides (17). In an earlier study, we showed increased opsonization in approximately 80 % of postvaccination sera with at least a twofold antibody increase to S. pneumoniae serotypes included in the vaccine.¹ The present investigation indicates that pneumococcal vaccination might produce protection to S. pneumoniae serotypes immunologically related to the vaccine serotypes. However, careful longterm studies on vaccinated human subjects are needed to clarify the degree of protection.

References

1. Youmans, G.P. *Streptococcus pneumoniae* (pneumococcus) in G.P. Youmans, P.Y. Paterson and H.M. Sommers (ed.), *The Biologic and Clinical Basis of Infectious Diseases* 2:nd ed., W.B. Saunders Co, Philadelphia, 1980, p. 330.
2. Lund, E., Henrichsen, J. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae* in T. Bergan and J.R. Norris (ed.). *Methods in Microbiology*, vol. 12, Academic Press Inc., New York, 1978, p. 241-262.
3. Austrian, R. Some observations on the pneumococcus and on the current status of pneumococcal disease and its prevention. *Rev. Infect. Dis.* 3:51-517, 1981.
4. Austrian, R., Douglas, R.M., Schiffman, G., Coetzee, A.M., Koornhof, H.J., Hayden-Smith, S., Reid, R.D.W. Prevention of pneumococcal pneumonia by vaccination. *Trans. Assoc. Am. Phys.* 89: 184-194, 1976.
5. Broome, C.V., Facklam, R.R., Allen, J.R., Fraser, D.W., Austrian, R. Epidemiology of pneumococcal serotypes in the United States 1978-1979. *J. Infect. Dis.* 141:119-123, 1980.
6. Robbins, J.B., Lee, C.-J., Rastogi, S.C., Schiffman, G., Henrichsen, J. Comparative immunogenicity of group 6 pneumococcal type 6A (6) and type 6B (26) capsular polysaccharides. *Infect. Immun.* 26:1116-1122, 1979
7. Szu, S.C., Lee, C.-J., Parke, J.C. Jr., Schiffman, G., Henrichsen, J., Austrian, R., Rastogi, S.C., Robbins, J.B. Cross-immunogenicity of pneumococcal group 9 capsular polysaccharides in adult volunteers. *Infect. Immun.* 35:777-782, 1982.
8. Lee, C.-J., Fraser, B.A., Szu, S., Lin, K.-I. Chemical structure of and immune response to polysaccharides of *Streptococcus pneumo-*

- niae. Rev. Infect. Dis. 3:323-331, 1981.
9. Appelbaum, P.C., Bhamjee, A., Scragg, J.N., Hallet, A.F., Bowen, A.J., Cooper, R.C. Streptococcus pneumoniae resistant to penicillin and chloramphenicol. Lancet 2:995-997, 1977.
 10. Radetsky, M.S., Istre, G.R., Johansen, I.L., Parmelee, S.W., Lauer, B.A., Wiesenthal, A.M., Glode, M.P. Multiply resistant pneumococcus causing meningitis: Its epidemiology within a day-care center. Lancet 2:771-773, 1981.
 11. Braconier, J.H., Odeberg, H. Granulocyte phagocytosis and killing of virulent and avirulent serotypes of Streptococcus pneumoniae. J. Lab. Clin. Med. 100:279-287, 1982.
 12. Braconier, J.H., Odeberg, H. Evaluation of phagocytic and bactericidal activities of neutrophil granulocytes. Determination of viable extracellular bacteria by their incorporation of ^{14}C -leucine and ^3H -thymidine. Scand. J. Haematol. 23:407-414, 1979.
 13. Odeberg, H., Olofsson, T., Olsson, I. Granulocyte function in chronic granulocytic leukemia. I. Bactericidal and metabolic capabilities during phagocytosis in isolated granulocytes. Br. J. Haematol. 29:427-441, 1975.
 14. McConehney, P.J., Dixon, F.J. A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy 29:185-189, 1966.
 15. Christensen, K.K., Christensen, P., Mårdh, P.-A., Weström, L. Quantitation of serum antibodies to surface antigens of Neisseria gonorrhoeae with radiolabeled protein A of Staphylococcus aureus. J. Infect. Dis. 134:317-323, 1976.
 16. Dorvai, G., Welsh, K.I., Wigzell, H. Labeled staphylococcal protein A as an immunological probe in the analysis of cell surface markers. Scand. J. Immunol. 3:405-411, 1974.

17. Winkelstein, J.A. Opsonins: their function, identity and clinical significance. *J. Pediatr.* 82:747-753, 1973.
18. Szu, S., Lee, C.-J., Carlo, D., Henrichsen, J. Immunochemical characterization of cross-reactivity of pneumococcal group 9 capsular polysaccharide types 9N, 9A, 9L, and 9V. *Infect. Immun.* 31: 371-379, 1981.
19. Harboe, M., Fölling, I. Recognition of two distinct groups of human IgM and IgA based on different binding to staphylococci. *Scand. J. Immunol.* 3:471-482, 1974.
20. Kronvall, G., Williams, R.C. Jr. Differences in anti-protein A activity among IgG subgroups. *I. Immunol.* 103:828-833, 1969.

Footnote

1. Pneumococcal vaccination: opsonic and antibody response in immunocompromized individuals to serotypes 6A, 19F and 23F. Braconier, J.H., Pedersen, F.K., Rosén, C.
Manuscript in preparation.

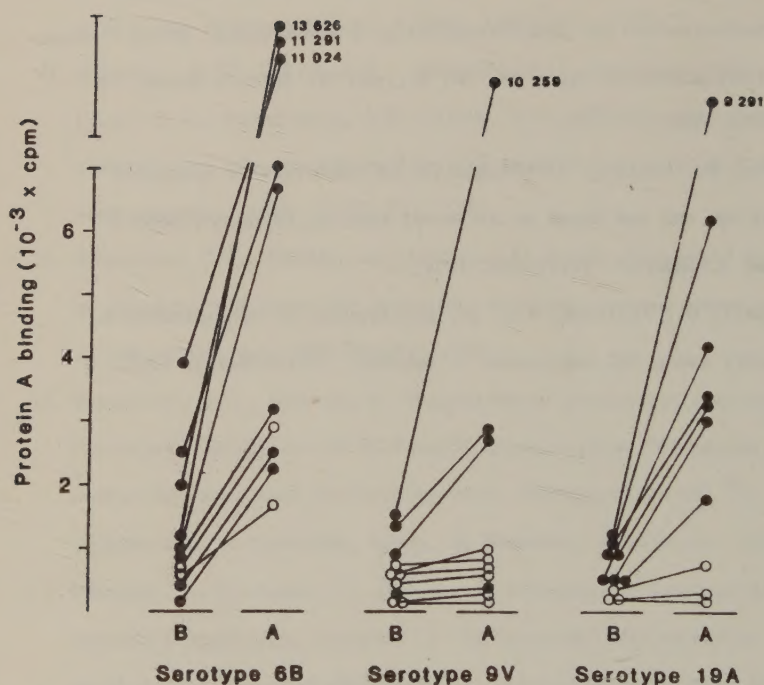


Fig. . Antibody and opsonic response to pneumococcal serotypes 6B, 9V and 19A before (B) and after (A) vaccination of humans with 14-valent pneumococcal vaccine. The antibody levels are expressed as the amount of radiolabeled protein A bound to live pneumococci pre exposed to diluted patient sera. Solid circles indicate serum pairs with increased opsonic activity after immunization and open circles denote stable opsonic activity.

